Purinergic signalling in the musculoskeletal system

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Abstract It is now widely recognised that extracellular nucleotides, signalling via purinergic receptors, participate in numerous biological processes in most tissues. It has become evident that extracellular nucleotides have significant regulatory effects in the musculoskeletal system. In early development, ATP released from motor nerves along with acetylcholine acts as a cotransmitter in neuromuscular transmission; in mature animals, ATP functions as a neuromodulator. Purinergic receptors expressed by skeletal muscle and satellite cells play important pathophysiological roles in their development or repair. In many cell types, expression of purinergic receptors is often dependent on differentiation. For example, sequential expression of P2X5, P2Y₁ and P2X2 receptors occurs during muscle regeneration in the *mdx* model of muscular dystrophy. In bone and cartilage cells, the functional effects of purinergic signalling appear to be largely negative. ATP stimulates the formation and activation of osteoclasts, the bone-destroying cells. Another role appears to be as a potent local inhibitor of mineralisation. In osteoblasts, the bone-forming cells, ATP acts via P2 receptors to limit bone mineralisation by inhibiting alkaline phosphatase expression and activity. Extracellular ATP additionally exerts significant effects on mineralisation via its hydrolysis product, pyrophosphate. Evidence now suggests that purinergic signalling is potentially important in several bone and joint disorders including osteoporosis, rheumatoid arthritis and cancers. Strategies for future musculoskeletal therapies might involve modulation

of purinergic receptor function or of the ecto-nucleotidases responsible for ATP breakdown or ATP transport inhibitors.

Keywords Bone · Cartilage · Joints · Arthritis · Muscular dystrophy · Cancer

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Introduction

A physiological role for ATP at the skeletal neuromuscular junction was recognised early. Injection of ATP into the sciatic artery supplying the gastrocnemius muscle of the frog produced tetanus-like contractions [1]. They also reported that the sensitivity of the preparation to acetylcholine (ACh) was greatly increased by previous application of ATP [2]. ATP was later shown to be released together with ACh from cholinergic nerves in various tissues, including the electric organ of elasmobranch fish [3, 4] and phrenic nerve endings in the rat diaphragm [5, 6]. However, it was not recognised at the time as a cotransmitter, but was considered as a molecule involved in the vesicular uptake and storage of the neurotransmitter ACh.

Investigations concerned with purinergic signalling in bone did not begin until the late 1980s. Initial studies demonstrated that extracellular nucleotides could induce increases in intracellular Ca^{2+} in bone cells [7–10]. Later, it was shown that extracellular ATP could inhibit bone formation whilst promoting bone resorption [11, 12]. There is now abundant evidence to show that extracellular nucleotides play a key role in modulating the differentiation, function and survival of bone cells.

In this article, we review in depth the role of purinergic signalling in mature and developing skeletal muscle, bone and cartilage in health and disease

Skeletal muscle

Skeletal neuromuscular junction

To date, much of the evidence for purinergic involvement in skeletal neuromuscular transmission has come from studies of the fish electric organ, frog and chick neuromuscular junctions (see [13] and [14–21]).

In early studies, ATP and adenosine were shown to inhibit the release of ACh from motor nerve terminals [22, 23]. The effect of ATP was due to adenosine, produced after ATP breakdown, acting on presynaptic A_1 receptors [24–28]. ATP also acted postsynaptically to facilitate the action of ACh [29]. In the early development of the neuromuscular junction, released ATP can act on P2X receptor ion channels as a genuine cotransmitter along with ACh acting on nicotinic receptors. However, in mature animals, ATP acts as a modulator at both pre-junctional and post-junctional sites, rather than as a cotransmitter [23, 30–34] (Fig. 1). It was also demonstrated that ACh and ATP were both released by exocytosis from synaptic vesicles [35, 36] and there was a high-affinity adenosine uptake system in synaptosomes for reconstitution of stored ATP. Excitatory adenosine $\text{A}_{2\text{A}}$ receptors also coexist with inhibitory (A_1) receptors at the rat and mouse neuromuscular junction [37–40].

P2X7-like receptors are expressed in the mouse neuromuscular junction [41] and activation of these receptors results in release of synaptic vesicles [42]. ATP, acting via P2Y receptors, was shown to inhibit non-quantal ACh release at the neuromuscular junction of mouse [43, 44]. Later, it was identified that activation of the P2Y_{12} and P2Y_{13} receptors, as well as adenosine A_1 receptors, inhibited ACh release from mammalian motor nerve terminals [45]. The role of P2Y receptors

Fig. 1 **a** In the adult skeletal neuromuscular junction, acetylcholine (ACh) is the neurotransmitter acting on postsynaptic nicotinic receptors. ATP is released during neurotransmission and, after breakdown to adenosine, acts on presynaptic $\text{P1}(\text{A}_1)$ receptors to inhibit release of ACh. **b** In the developing myotube, ATP is a genuine cotransmitter together with ACh acting on postsynaptic P2X receptors. (Reproduced from [13] with permission from Springer)

in ATP-mediated regulation of non-quantal ACh release from motor nerve endings in the rat diaphragm has recently been reviewed in detail [46]. A study detailing the reversal effect of NF449, an analogue of suramin, on neuromuscular blockade induced by d-tubocurarine has been published [47]. ATP co-released with ACh from motor nerve terminals may also play a role in the regulation of the cell volume of muscle fibres [48].

During development, expression of P2X2 receptors coincides with the appearance and formation of neuromuscular junctions [49]. This receptor interacts with nicotinic ACh receptors to produce cross-inhibition of channel opening [50]. Using the P2X2 receptor knockout mouse, it was shown that in the absence of P2X2 receptors, the post-junctional synaptic folds rich in nicotinic receptors were disorganized; there was also misapposition of pre-synaptic terminals and regions of high localization of nicotinic receptors [51]. Furthermore, there was decreased density of post-synaptic junctional folds and increased end-plate fragmentation. ATP promotes stabilization of the neuromuscular junction and may play a role in activity-dependent synaptic modifications during development [52].

Sensory fibres originating in the dorsal root ganglia (DRG) supply skeletal muscle, and it was concluded that P2X5 and/or P2X4 receptors in combination with proton-activated channels and transient receptor potential cation channels are used to detect metabolites produced by contracting muscles [53]. The exercise pressor reflex is evoked by contraction and it was proposed that P2X3 and P2X2/3 receptors on muscle afferents mediate this reflex [54].

Skeletal muscle

In the early 1980s, extracellular ATP was shown to act on skeletal muscle cells when it was suggested that cultured myoblasts and myotubes may have ATP-sensitive cation channels [30]. It was shown that when the ATP analogue adenylyl-5'-yl imidodiphosphate was added to muscle fibres in rigor, it caused the fibre to lengthen, but not apparently by acting at the contractile actin myofilament level (see [55]). The source of ATP can be as a cotransmitter released from motor nerves or it can be released from stressed or exercising muscle, as well as damaged or dying cells and vascular endothelial cells [56–58]. Extracellular ATP, acting via P2 receptors, was later shown to induce inositol 1,4,5-trisphosphate (InsP₃) accumulation before or after fusing rat skeletal muscle cells in culture, but not during the period of fusion [59]. Nucleotide diphosphates also activated ATP-sensitive potassium channels in mouse skeletal muscle [60]. Two types of human P2Y₁ cDNA clones were isolated from the human erythro leukaemia cell cDNA library. A multiple human tissue Northern blot indicated two transcripts of approximately 4.4

and 7.0 kb and the mRNA for both transcripts were expressed in skeletal muscle [61].

A P2X receptor gene, regulated by p53, was shown to be expressed predominantly on skeletal muscle and it was suggested that the receptors may play a role in the proliferation and/or differentiation of skeletal muscle [62]. In addition, it was also suggested that altered expression may be involved in the development of some sarcomas [62]. Novel P2X receptors were also described on chick embryos [63, 64], although it was later shown to be homologous to the P2X5 receptor isoform [65]. In cultures of human skeletal muscle fibres, ATP was shown to elevate intracellular Ca²⁺ ([Ca²⁺]_i) [66]. The response to α,β -methylene ATP (α,β -meATP) was greater than ATP, suggesting that the effects were mediated by a P2X1 and/or P2X3 receptors.

The pattern of purinergic receptor expression in skeletal muscle varies with the developmental stage and species. Sequential expression of P2X2, P2X5 and P2X6 receptors has been described for developing rat skeletal muscle [49], while P2X1, P2X4, P2X5 and P2X6 receptors have been demonstrated in developing chicken myoblasts [65, 67, 68]. One study suggested that P2X4 receptors are localised intracellularly on T-tubule membranes, suggesting that they may play a role in excitation–contraction coupling [69]. Thus, P2X receptors play important roles in the early development of skeletal muscle, but immediately after birth, the expression levels of P2X receptors are reduced and often functionally undetectable [67, 70]. Low levels of P2X7 receptors were detected in human skeletal muscle [71]. ATP also regulates the differentiation of mammalian skeletal muscle by activation of P2X5 receptors on satellite cells [72]. P2X4, P2X5, P2X7, P2Y₁ and P2Y₄ receptors have also been demonstrated on multinucleated mouse myoblasts [73].

A number of studies have also implicated P2Y receptors in skeletal muscle development. P2Y receptors were described on developing C2C12 mouse myotubes [74] and on embryonic skeletal muscle [75]. P2Y₁, P2Y₂ and P2Y₄ receptors were shown to be only temporarily expressed in embryos and were down-regulated in postnatal life. Differentiating skeletal muscle C2C12 cells express P2Y₁, P2Y₂, P2Y₄ and P2Y₆ receptor mRNA [70]. At the transcriptional level, P2Y₁ and P2Y₂ receptors have been identified in human skeletal muscle [76, 77] and ATP stimulates an increase in [Ca²⁺]_i in human myotubes [78]. 5'-Guanosine-triphosphate (GTP) has also been shown to play a role in the early phases of skeletal muscle differentiation [79]. However, whilst purinoceptors clearly play significant roles during the development of skeletal muscle, differentiated muscle fibres barely express them, but after denervation they reappear [14]. The role of purinergic signalling during the formation of skeletal muscle myotubes has also been comprehensively examined [80].

A number of studies have investigated the intracellular signalling pathways activated following stimulation of P2

receptors on skeletal muscle. Treatment with ATP significantly increased the strength of mouse diaphragm muscle contractions induced by carbachol and suramin abolished this effect, as did the specific protein kinase C (PKC) inhibitor, chelerythrine [81]. Differentiation of skeletal muscle cells both in vivo and in vitro is accompanied by the development of alterations of voltage-dependent processes and alterations in purinergic signalling [82]. Overexpression of PKC α demonstrated the appearance of increases in $[Ca^{2+}]_i$ elicited by ATP, but failed to do so after depolarising stimuli. PKC α over-expression changed the purinergic receptor pattern of immature myoblasts; there was lack of increase in the expression of P2X7 receptors and failure of P2Y₄ receptors to appear and P2Y₂ receptors to disappear. Uridine 5'-triphosphate (UTP) produced InsP₃-dependent Ca^{2+} release in cultured myotubes [83], but UTP had little effect on adult muscles [84].

Ca^{2+} influx into C2C12 cells via P2X receptors contributes to phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) [70]. In differentiated human skeletal muscle cells, ATP evoked a Ca^{2+} transient, which was mediated by P2Y receptors leading to the activation of ERK1/2. This suggests that extracellular ATP participates in skeletal muscle plasticity and highlights the importance of ATP as a cotransmitter at the neuromuscular junction via dual signalling, causing InsP₃ receptor-mediated Ca^{2+} transients and Ca^{2+} -insensitive phosphorylation of ERK1/2 [85].

The P2Y₁ receptor is emerging as having an important role in skeletal muscle function. Extracellular nucleotides, acting via the P2Y₁ receptor, enhance muscle contraction by increasing the expression of ACh receptors and the activity of cholinesterase [86]. Activation of this receptor by ATP also inhibits Cl^- channels in mature mammalian skeletal muscle; this has implications for muscle excitability and fatigue and the pathophysiology of myotonias [87]. Upregulation of Na,K-ATPase activity during muscle activity appears to be due to the stimulatory effect of adenosine 5'-diphosphate (ADP) acting via P2Y₁₃ receptors with some contribution from P2Y₁ receptors [88]. The signalling via P2Y₁ receptors to increase the Na^+ - K^+ pump activity improves the force and excitability of depolarised skeletal muscle, which may be important for the maintenance of excitability during intense exercise [89]. Supplementation with ATP for 15 days was reported to reduce muscle fatigue at the end of an exhaustive exercise bout [90].

ATP is released from skeletal muscle during titanic contraction [56, 69] and high-frequency electrical stimulation leads to fatigue involving extracellular Ca^{2+} ; however, it was claimed that P2X receptor antagonists did not affect the development of high-frequency fatigue [91]. ATP is released by electrical stimulation of skeletal muscle, probably through pannexin hemichannels [92, 93], eliciting both a fast calcium signal associated with contraction via P2X receptors and a slow signal that regulates gene expression via P2Y receptors

[56] and triggers interleukin (IL)-6 expression [94]. ATP is also released from skeletal muscle by mechanical stimulation (compression) [95].

ATP, released by autocrine/paracrine mechanisms, also regulates proliferation of C2C12 myoblasts [96]. A recent paper demonstrated that extracellular ATP controls mouse myoblast proliferation via induction of reactive oxygen species [97]. Activation of P2X receptors on skeletal muscle appears to play a role in mediating the exercise pressor reflex [98]; later, it was suggested that the ATP that triggered this reflex was released from contracting muscle [99] or endothelial cells [57].

Cytoplasmic Ca^{2+} concentration and Ca^{2+} handling molecular machinery tightly regulate skeletal muscle contraction and excitation of skeletal muscle results in rapid Ca^{2+} release from sarcoplasmic reticulum through the ryanodine receptor channel type 1 (RyR1). Nucleotides activate skeletal muscle ryanodine receptors, whilst adenosine inhibits the ATP activation of these receptors [100]. In addition, a P2 receptor antagonist, the suramin analogue, NF676, inhibits the RyR1 receptor [101]. The ATP binding characteristics of RyR1 are modulated by a variety of pharmacological agents, and it was speculated that modulation of ATP binding may play a central role in the regulation of the open/closed state of the channel [102]. It is now apparent that the transient component of calcium flux depends on activation of P2X receptors and the consequent depolarisation, whereas P2Y receptors are responsible for most of the maintained calcium flux [103].

The synthesis of extracellular ATP was found to be increased with a high level of adenylate kinase 1 (AK1) and ATP synthase β during C2C12 myogenesis [104]. Experiments with AK1 knock-down led to the conclusion that AK1 secretion is required for ATP generation in myotubes. Extracellular ATP increased L-carnitine transport and content in C2C12 cells; specific inhibitors of P2X and P2Y receptors abolished this effect [105].

In terms of P1 receptor signalling, a dramatic increase in expression of A_{2B} and A₃ (but not A₁ or A_{2A}) receptors was observed following traumatic skeletal muscle injury. Thus, it was suggested that both receptors may be potential targets for stimulation of skeletal muscle regeneration and functional restoration after injury [106]. In addition, a protective role for adenosine, acting via A₃ receptors, was identified for the treatment of post-traumatic skeletal muscle injury [107].

Bone

Within the skeleton, osteoblasts are the cells responsible for bone formation, whilst osteoclasts control bone resorption. A balance between osteoblastic bone formation and osteoclastic resorption is essential for a functional skeleton. In vivo mechanical loading can induce new bone formation and

increase bone mineral density, whereas disease, immobilization and weightlessness can all lead to bone loss. Osteocytes, which are the most abundant cell type in bone, form an interconnecting network concerned with sensing mechanical load. Chondrocytes are the cells responsible for cartilage formation.

It was recognised in the 1990s that ATP released from damaged cells or by physiological secretion in response to mechanical stress could be an important factor in the remodelling of bone [108, 109]. Since these early investigations, understanding about the regulation of bone cell function by purinergic signalling has increased considerably.

Many studies have demonstrated the expression of multiple P2X and P2Y receptor subtypes in osteoclasts, osteoblasts and chondrocytes from a number of species, including rat, mouse, rabbit and human (see reviews [110–113]). Analysis of P2Y₁, P2Y₂, P2Y₆, P2Y₁₂, P2Y₁₃ and P2X₇ knockout mice has provided evidence for the important role of purinergic signalling in bone remodelling *in vivo* [115–122]. Previous reviews concerned with purinergic signalling on bone have focussed on different aspects, including bone marrow cells [123]; bisphosphonates [124–126]; osteoblasts and osteoclasts [113, 127–129]; bone remodelling [110, 130–132]; osteoblasts and antiresorptive drugs [133–135]; P2X₇ receptors [136–139]; neuroskeletal signalling pathways [140]; exercise and mechanical loading of bone [141]; calcium signalling in bone [142]; adenosine in inflammatory joint diseases [143]; P₂ receptor knockout mice and bone phenotypes [118]; ion channels and transporters in osteoclasts [144]; platelets and bone reconstruction therapy [145]; bone mineralisation [146]; acidosis and hypoxia [147]; and adenosine receptors in rheumatoid arthritis (RA) [148].

Osteoblasts

P2 receptor expression by osteoblasts

The first description of purinergic signalling in osteoblasts was in the late 1980s when P₁ (A₂-like) adenosine receptors were reported on osteoblasts [149, 150]. Increases in [Ca²⁺]_i of osteoblasts in response to ATP and UTP were later described [7–9, 109], indicating the presence of more than one subtype of P2Y receptor [151–155].

P2U (i.e. P2Y₂ and/or P2Y₄) receptors were cloned from human osteoblasts in the 1990s [156]. In single cell studies of human osteoblasts, different populations responded predominantly to ADP, others to ATP or UTP; this was suggested to reflect variations in the differentiation status of individual cells [157]. This idea was confirmed by a later study, which demonstrated that osteoblastic expression of P₂ receptors was strongly dependent on cellular differentiation [158]. A higher expression of P2Y₂ receptors was also found in differentiated compared to undifferentiated MC3T3-E1 osteoblasts [159].

Furthermore, it was recently shown that the P2Y receptor-mediated increase in [Ca²⁺]_i from the osteoblastic TBR31-2 cell line differs with time in culture and is associated with the level of expression of P2Y receptors [160].

A number of studies have now demonstrated the expression of P2Y₁, P2Y₂, P2Y₄ and P2Y₆ receptor mRNA and protein in several human osteoblast-like cell lines (MG-63, OHS-4, SaM-1, SaOS-2, Te85) [156, 161, 162] and the rat osteosarcoma cell line, UMR106-01 [163, 164]. Of the several P2Y receptors expressed by the UMR106-01 cell line, the P2Y₁ receptor is thought to be predominant [163]. In contrast, another rat osteosarcoma cell line ROS17/2.8 does not appear to express functional P2Y₂ receptors [164]. Functional P2Y receptors, particularly the P2Y₁ subtype, were also shown to be expressed by human G292 osteoblastic cells [165]. Primary rat calvarial osteoblasts were initially shown to express mRNA and protein for the P2Y₁, P2Y₂, P2Y₄ and P2Y₆ receptors [158, 166]. Later studies also reported the expression of the P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors in this model [167, 168].

Widespread expression of P2X receptors on osteoblasts has also been reported. Initial studies on cultured rat osteoblasts identified the expression of the P2X₂ and P2X₅ receptor subtypes [166]. Follow-up studies by the same group also demonstrated the expression of mRNA and protein for the P2X₁, P2X₃, P2X₄ and P2X₆ receptor, although there were changes in expression during differentiation [158, 167]. This study also demonstrated large variations in the relative expression of the P₂ receptors in osteoblasts, with mRNA for the P2X₄ receptor being the most abundant [167]. Expression of the P2X₇ receptor was first demonstrated in a subpopulation of human differentiated osteoblasts [169]; later studies also demonstrated expression in rat [158, 167] and mouse osteoblasts [137]. Interestingly, a recent study claimed that P2X₇ receptors are localised in caveolae in the plasma membranes of osteoblasts [170]. Expression of multiple P2X receptor subtypes has also been shown on several human osteoblast-like cell lines (MG-63, SaOS-2, SaM-1) [162, 171, 172].

Functional effects of P2 receptor-mediated signalling in osteoblasts

Extracellular nucleotides acting via P₂ receptors have been shown to exert numerous effects on osteoblast proliferation, differentiation, function, gene expression and cell signalling. The mitogenic effects of ATP were first reported by Suzuki et al. who showed that the ATP-stimulated Ca²⁺ influx resulted in synthesis of prostaglandin E₂ (PGE₂), which contributed to the ATP-induced proliferative action [155]. Subsequently, ATP acting via the P2X₅ receptor was shown to promote DNA synthesis in human osteoblast-like MG-63 cells [171]. Adenosine was also reported to have mitogenic actions on MC3T3-E1 osteoblast-like cells, stimulating

DNA synthesis and enhancing platelet-derived growth factor-induced mitogenesis [150].

A number of investigations have examined the intracellular signalling pathways activated in osteoblasts following P2Y receptor stimulation. An early study reported that ATP stimulated phospholipase (PL) D in a Ca^{2+} /calmodulin-dependent manner in osteoblasts, with neither PKC activation nor GTP-binding protein being involved [173]. More recently, it was shown that P2Y₂ receptor-mediated stimulation of osteoblasts involved activation of ERK 1/2 and p38 mitogen-activated protein kinase (MAPK) pathways [174]. P2Y₂ receptor stimulation by ATP has also been shown to induce JNK1 phosphorylation in osteoblast-like cells, and this signalling mechanism was suggested to play a role in the bone remodelling induced by ATP since it could stimulate osteoblast proliferation [175]. The proliferative effect of ATP involves the participation of the PI3K/Akt signalling pathway in rat osteoblasts [176] and also in differentiation induced by ATP and UTP via P2Y₂ receptors [177]. Furthermore, G protein-coupled receptor kinase 2 inhibits ATP-induced activation of the ERK1/2 signalling pathway in MC3T3-E1 osteoblastic cells, and this was suggested to play a role in mechano-desensitization in osteoblasts [178].

ATP stimulates the early growth response protein 1 via a PKC-dependent pathway in human osteoblast-like HOBIT cells [179]. Runx2, a bone-related transcription factor required for osteoblast differentiation and bone formation, is activated by ATP and UTP in HOBIT cells. It was suggested that this underlies the link between mechanical stress and the response of osteoblasts, through release of nucleotides [180]. Pre-stress in the cytoskeleton of cells affects the magnitude of cellular responses to mechanical stimuli. ATP was shown to reduce pre-stress by monitoring the rate of collagen gel compaction [181] via MAPK and nuclear factor κB (NF- κB) pathways [182]. A novel Ca^{2+} -activated K^+ channel (IK) is expressed by human osteoblasts, and ATP-induced hyperpolarization was inhibited by IK channel blockers [183]. Using green fluorescent-labelled paxillin fusion protein as a marker, it was shown that stimulation of osteoblasts with extracellular ATP induced the formation of short-living membrane protuberances [184].

Purinergic signalling can interact with other intracellular pathways to regulate osteoblast function. In early studies, P2 receptors were shown to mediate the potentiation of parathyroid hormone receptor-mediated increases in $[\text{Ca}^{2+}]_i$ and InsP_3 in UMR-106 rat osteoblasts [185, 186]. Synergistic co-activation by ATP acting via P2Y receptors and parathyroid hormone was found to increase mRNA levels for *c-fos*, a transcription factor with a pivotal role in osteoblastic activation and bone remodelling. It was suggested that this may be a mechanism to generate strong localised responses to systemic growth and differentiation factors [187]. In a later study, it was shown that parathyroid

hormone potentiated nucleotide-induced $[\text{Ca}^{2+}]_i$ release in rat osteoblasts independently of G_q activation or cyclic adenosine monophosphate (cAMP) accumulation [163]. However, in a more recent study of MC3T3-E1 osteoblastic cells, parathyroid hormone enhanced bone morphogenetic protein activity by increasing intracellular cAMP accumulation to promote new bone formation [188]. Additionally, the regulator of G protein signalling protein 2 in osteoblasts was upregulated by both extracellular ATP and parathyroid hormone and this contributes to cross-desensitization of G_s- and G_q-coupled signals [189].

It has been demonstrated that ATP can induce the release of IL-6 [162], arachidonic acid via a PLD pathway [190] and synergise with glucose-dependent insulinotropic polypeptide to induce *c-fos* in osteoblastic cells [191]. There was also early recognition that locally released ATP could set up calcium waves between osteoblasts, mediated by P2Y₂ receptors and connexin 43 hemichannels [164].

P2Y receptors have been implicated in the oscillatory fluid flow-induced intracellular Ca^{2+} mobilization in osteoblastic cells [192]. Furthermore, oscillating fluid flow activation of gap junction hemichannels between osteocytes and osteoblasts induces the release of ATP from MLO-Y4 osteocytes [193]. P2Y receptors and gap junctions are also involved in the propagation of intercellular calcium waves during osteoblast differentiation in vitro [194]. Calcium wave propagation was significantly impeded when extracellular ATP, released in response to mechanical stimulation, was hydrolysed by apyrase. This implied that ATP diffusion, rather than transport through gap junctions, is the underlying mechanism, involving P2Y receptors and release of Ca^{2+} from intracellular stores [195, 196]. A study also demonstrated that cyclic hydraulic pressure and fluid flow differentially modulate cytoskeleton re-organization in MC3T3 osteoblasts. This was suggested to contribute to the loss of mechanosensitivity with extended loading [197].

Reactive oxygen species, such as H_2O_2 , mediate $[\text{Ca}^{2+}]_i$ activity in primary rat osteoblasts by increasing Ca^{2+} release from intracellular stores [198], through sensitization of P2Y₂ receptors [199]. It was suggested that mild oxidative conditions, represented by H_2O_2 , could activate purinergic signalling through the sensitization of P2Y₂ receptors [200]. ATP was claimed to induce mineralisation of MC3T3-E1 osteoblast-like cells and three phosphatases acted synergistically to promote ATP-mediated mineralisation [201]. However, a number of other investigations have observed the opposite effect. Initial functional studies showed that low micromolar concentrations of ATP and UTP acting via P2Y₂ receptors inhibited bone formation by cultured rat osteoblasts [11, 202]. A follow-up investigation demonstrated that ATP and UTP selectively inhibited the mineralisation of the organic matrix and provided further evidence for involvement of the P2Y₂ receptor subtype [203]. Subsequent skeletal analysis of

P2Y₂ receptor knockout mice by dual energy X-ray absorptiometry (DEXA) and microcomputed tomography (μCT) demonstrated large increases in trabecular and cortical bone parameters in the long bones [117, 204].

Recently, it was also demonstrated that the P2X₁, P2X₃ and P2X₇ receptor agonists, α,β-meATP, β,γ-methylene ATP and 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (Bz-ATP) reduced bone mineralisation in vitro [167]. This study used selective P2 receptor antagonists to suggest that the effects were mediated via the P2X₁ and P2X₇ receptor subtypes [167]. Furthermore, a recent investigation demonstrated increased bone mineralisation in osteoblast cultures treated with apyrase, suggesting that endogenous ATP released by osteoblasts acts as an important local brake on mineralisation [205]. Combined these data suggests that ATP acts as a key endogenous inhibitor of bone mineralisation.

Clopidogrel (Plavix®) is a selective P2Y₁₂ receptor antagonist. It is an antithrombotic prescribed to reduce the risk of heart attack and stroke, acting via inhibition of platelet aggregation. Low micromolar concentrations of clopidogrel have been shown to inhibit osteoblast proliferation, differentiation and function and reduce cell viability [168]. Reduced trabecular bone was also observed in ovariectomised mice treated with clopidogrel for 4 weeks [168]. In contrast, a paper by Su et al. [120] demonstrated increased bone in mice treated with clopidogrel. However, the two investigations used very different dosing regimes (oral dosing compared to clopidogrel-treated water), and thus, the divergent results may reflect variations in the final dose received by the test subjects. In agreement with this idea, a study looking at a cohort of Danish patients found that clopidogrel was associated with fracture risk; however, these effects were biphasic with high doses (the recommended range) being associated with increased fracture risk and low doses being associated with decreased fracture risk [206].

Studies using the P2Y₁₃ receptor knockout mouse model demonstrated reduced trabecular bone mass, decreased osteoblast numbers in vivo and a reduced rate of bone remodelling indicating a role for this receptor in bone turnover [121]. The P2Y₁₃ receptor has also been shown to play an important role in the terminal differentiation of osteoprogenitor cells into osteoblasts or adipocytes [207].

Despite expressing all P2X receptor subtypes, the effects of P2X receptor activation on osteoblasts remain poorly investigated. The main exception being the P2X₇ receptor, which has been the focus of numerous studies often with conflicting results. Early investigations demonstrated that functional P2X₇ receptors expressed by a subpopulation of human differentiated osteoblasts mediated apoptotic cell death [169, 208]. Functional studies of P2X₇ receptor activation have shown increased [209] and decreased [167] bone formation in response to Bz-ATP. P2X₇ receptors mediate blebbing in osteoblasts via a pathway involving lysophosphatidic acid,

and it was suggested that this pathway may contribute to P2X₇ receptor-stimulated osteogenesis during skeletal development and mechanotransduction [209, 210].

P2X₇ receptor antagonists significantly inhibit ATP release from osteoblasts [211] and *c-fos* activation in response to fluid flow [212]. Fluid shear stress can also regulate gene expression in osteoblasts, in part by activation of the transcription factor NF-κB, through P2X₇ and P2Y₆ receptors [213]. Brief activation of P2X₇ receptors on MC3T3-E1 osteoblast-like cells has been shown to trigger a dramatic Ca²⁺-dependent stimulation of metabolic acid production [214]. It was proposed that an acidic zone beneath the active osteoblast layer may prevent premature mineralisation of the osteoid seam during bone formation or that acid production by cells of the osteoblast lineage may activate osteoclastic resorption. It has also been reported that P2X₇ receptors play a role in ovariectomy-induced bone loss in mice [215] and callus remodelling during fracture repair [216].

It has been suggested that ATP, perhaps via P2X₇ receptor activation, mediates prostaglandin synthesis in response to fluid shear and may contribute to the response of bone to mechanical loading [138, 217]. Furthermore, fluid shear-induced ERK1/2 phosphorylation requires Ca²⁺-dependent ATP release and mediation via P2X₇ (but not P2Y₂) receptors [218, 219]. Additionally, it has been claimed that the P2X₇ receptor is involved in propagation of calcium waves in mature osteoblasts [208].

A number of studies using P2X₇ receptor knockout mice from different genetic backgrounds have been performed. The initial study by Ke et al. [137] reported a decreased bone mineral content and periosteal circumference accompanied by decreased bone formation parameters and increased bone resorption parameters [137]. A separate study by Gartland et al. [136] reported no differences in bone mineral density of trabecular bone, but an increase in cortical bone was observed. It was subsequently reported that the knockout model used by Gartland et al. expresses a P2X₇ splice variant in some tissues [220]; therefore, the results from this model should be interpreted with caution. Further complicating the analysis of the P2X₇ receptor knockout mouse model, a recent study demonstrated that the genetic background of the mouse strain used strongly influences the bone phenotype [122]. Gain of function polymorphisms of the P2X₇ receptor have been associated with increased bone mass [221], whilst loss of function polymorphisms have been associated with increased fracture risk, reduced bone mineral density and osteoporosis [222–224].

A number of P2 receptors (P2X₂, P2X₃, P2X₄, P2X₆, P2Y₄, P2Y₆ and P2Y₁₄) expressed by osteoblasts still have no known functional effects and present interesting areas for future study. Of particular note is the P2X₄ receptor, which displays the highest expression of all the P2 receptors present on osteoblasts [167]. Furthermore, given that

osteoblasts express all P2X receptor subtypes, it is possible that they may combine to form functional heteromultimers. Heteromultimers often display the pharmacology of both participating subunits, thus increasing the diversity of ATP-mediated signalling and downstream functional effects. At present, it is not known whether the presence of such heteromultimers would influence purinergic signalling in osteoblasts.

ATP release from osteoblasts

Controlled ATP release from osteoblasts was first described by Romanello et al. in 2001 [225]. Now a widely investigated area, there are many studies which indicate that the primary method of ATP release from osteoblasts is by vesicular exocytosis [217, 226–228]. However, it has also been suggested that maxi-anion channels [229] and the P2X7 receptor [211] may be involved in release of ATP from osteoblasts. Furthermore, a recent study has claimed that pannexin 1 channels, in concert with P2X7 receptors, form a molecular complex that underlies the hemichannel function in osteoblast mechanosignalling involving thaw release of ATP [230]. Ahnak, a 700-kDa scaffold protein, has also been shown to regulate ATP release and calcium signalling in osteoblasts in response to mechanical stimulation [231]. Evidence now suggests that controlled ATP release from osteoblasts leads to activation of sensory nerves via P2X receptors [232].

The amount of ATP released from osteoblasts depends on their differentiation state, with mature, bone-forming cells releasing up to sevenfold more than immature, proliferating cells [228]. Several studies have demonstrated increased ATP release in response to different external stimuli including hypoxia [228], mechanical stress [233] and fluid flow [217, 225]. Recently a detailed study was undertaken on ATP release in response to different forms of mechanical stimulation of human osteoblast SaOS-2 cells, including turbulent fluid flow, laminar fluid flow, substrate strain and 3D compressive loading [234]. It was concluded that the concentration of ATP released in response to mechanical loading varied in a time-, direction- and strain-dependent manner representing a local mechanostat in bone that may influence osteogenesis.

Osteoblasts are a target for calcitropic hormones, including the steroid $1\alpha,25(\text{OH})_2$ vitamin D_3 (1,25D). 1,25D induces a regulated secretion of ATP in osteoblasts, in the absence of mechanical stimulation, through interaction with vitamin D receptors and potentiation of Ca^{2+} and Cl^- signals required for vesicle fusion and exocytotic release [235]. 1,25D also induced exocytotic release from osteoblastic ROS 17/25 cells [236].

Ultrasound causes ATP release by osteoblasts *in vivo*, and it is suggested that this may contribute to accelerated fracture healing by enhancing osteoblast proliferation, increasing receptor activation of NF- κ B ligand (RANKL) expression and decreasing osteoprotegerin expression by osteoblasts to

promote osteoclastogenesis [237]. More recently, low-intensity pulsed ultrasound (LIPUS) treatment was shown to induce the release of ATP from osteoblasts and increase P2Y₁ receptor expression leading to proliferation [238]. The authors suggested that this underlies the mechanism whereby LIPUS leads to accelerated fracture healing.

In osteoblast-like cells, bisphosphonates, used for treatment of osteoporosis, have been reported to induce ATP release which then acts via P2Y receptors to activate ERKs [239].

ATP hydrolysis and the effects on bone mineralisation

Once it is released, ATP is rapidly broken down by a hydrolysis cascade involving a number of ecto-nucleotidases. Molecular and functional characterisation has shown there are four families of ecto-nucleotidases: (1) the NTPdases (ecto-nucleoside triphosphate diphosphohydrolase); (2) the NPPs (ecto-nucleotide pyrophosphatase/phosphodiesterase); (3) alkaline phosphatases; and (4) ecto-5'-nucleotidase [240]. Many ecto-nucleotidases have overlapping specificities. For example, NTPdases catalyse the reactions $\text{NTP} \rightarrow \text{NDP} + \text{phosphate (P}_i\text{)}$ and $\text{NDP} \rightarrow \text{NMP} + \text{P}_i$, whereas NPPs hydrolyse $\text{NTP} \rightarrow \text{NMP} + \text{pyrophosphate (PP}_i\text{)}$ or $\text{NDP} \rightarrow \text{NMP} + \text{P}_i$.

An early study using human SaOS-2 osteoblast-like cells showed coexistence of both ATP-consuming and ATP-generating activities on the cell surface [241]. Expression of multiple NTPdases and NPPs by osteoblasts has now been reported [203, 204]. Of these, NPP1 has emerged as having a particularly important role in bone. NPP1 converts extracellular ATP into AMP and PP_i , the potent inhibitor of bone mineralisation. A recent detailed study of the mouse model lacking NPP1 showed that this enzyme is essential for normal bone development and control of physiological bone mineralisation [242]. It is now becoming apparent that extracellular ATP is a key source of PP_i in bone [205]. Thus, it is likely that nucleotide triphosphates exert a dual inhibitory action on bone mineralisation via both P2 receptor-mediated signalling and direct hydrolysis to PP_i [111, 203, 205] (Fig. 2).

P1 receptors, adenosine and osteoblasts

The role of adenosine and P1 receptor-mediated signalling has become an area of increasing interest (see detailed review by [243]). An important early study showed that human osteoblast precursors produced extracellular adenosine, probably via breakdown of released ATP, which modulated their secretion of IL-6 and osteoprotegerin [244].

Human osteoblasts progenitor HC1 and bone marrow stromal (BMS) cells also show ecto-5'-nucleotidase (CD73) activity and express all four adenosine receptor subtypes, stimulation of which led to an increase in release of IL-6, which promotes osteoblastogenesis. Agonists to A₁ and A_{2A} receptors promote proliferation of osteoprogenitor and BMS cells, while

Fig. 2 Overview of the known functional effects of ATP on bone cells. ATP released from osteoblasts, osteocytes and osteoclasts can act locally to inhibit bone mineralisation and stimulate osteoclast formation and activity. ATP breakdown products can also act to influence bone cell function

A_{2B} receptor agonists promote differentiation [245]. Recently, CD73 knockout mice were used to show a potential role for adenosine in osteoblast differentiation [246]. Whilst another paper, using A_{2B} receptor knockout mice, identified this receptor as mediating the differentiation of mesenchymal stem cells into osteoblasts [247]. Adenosine was also shown to afford some protection against the death of MC3T3-E1 osteoblasts exposed to H_2O_2 -induced oxidative stress [248].

Osteocytes

Osteocytes account for about 90 % of all bone cells, yet little is known about the role of purinergic signalling in their survival and function. The primary purpose of osteocytes appears to be sensing mechanical load. They form an interconnected cell network located in the fluid-filled lacunocanalicular system, an ideal position to translate mechanical signals into biochemical signals to effector cells, to facilitate regulation of bone remodelling. When this osteocyte network was exposed to a steady fluid flow, calcium transients were recorded that were reduced by both suramin and thapsigargin [249], suggesting that this was due to ATP acting via P2Y receptors. Osteocytes express T-type voltage-sensitive calcium channels; the $\alpha\delta_1$ auxiliary subunit of which regulates mechanically-induced release of ATP from osteocyte cells [250]. Given that ATP can inhibit bone mineralisation, it is conceivable that release

from osteocytes *in vivo* could act to reduce progressive, age-related mineral encroachment from the surrounding bone (to which they have a very high surface area of exposure), thus preventing eventual cell ‘fossilisation’ and death (see Fig. 2).

Recently, RT-PCR and pharmacological analysis showed that P2Y₂ and P2X₇ receptors were functionally expressed in MLO-P2Y₄ osteocytes as well as the ability of these cells to release ATP upon mechanical stimulation [251] and UTP stimulation [252].

Osteoclasts

P2 receptor expression by osteoclasts

Osteoclasts, the bone-resorbing cells, are usually multinuclear and are formed from mononuclear progenitors of the monocyte/macrophage lineage. The first report of P2 receptor expression by osteoclasts came in the early 1990s, when it was shown that ATP induced a large, fast and transient increase in $[Ca^{2+}]_i$ in rabbit osteoclasts. This effect was inhibited by thapsigargin implying mediation by P2Y receptors [10]. Activation of the osteoclast ATP receptor was later shown to cause a transient decrease in intracellular pH [253]. The human P2U (P2Y₂/P2Y₄) receptor was subsequently cloned and sequenced from osteoclastoma, indicating that this receptor was probably also expressed by osteoclasts [156]. Early

studies also provided evidence for the presence of both P2X receptors, giving rise to non-selective cation currents, and P2Y receptors, mediating Ca^{2+} release from intracellular stores causing activation of a Ca^{2+} -dependent K^+ channels [254, 255]. P2X4 receptors were initially claimed to mediate ATP-activated non-selective cation currents in rabbit osteoclasts [256]. However, it was later found that the nucleotide-induced elevation of $[\text{Ca}^{2+}]_i$ arose primarily from activation of P2Y rather than P2X4 receptors [257].

Expression of multiple P2 receptor subtypes by osteoclasts has now been reported. Initial studies using rat long bone-derived osteoclasts showed expression of mRNA and protein for the P2X2, P2X4, P2X7, P2Y₁ and P2Y₂ receptors [166]. Human osteoclasts generated from peripheral blood were later shown to express the P2X1, P2X4, P2X7, P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ receptors [258]. Recently, osteoclasts derived from mouse bone marrow were shown to express all P2 receptors, with the exception of the P2X6 and P2Y₄ receptors [119]. This study also demonstrated large differences in the expression levels of the different P2 receptors, with the P2Y₆ receptor being the most abundant [119].

Functional effects of P2 receptor-mediated signalling in osteoclasts

Involvement of P2 receptors in the regulation of osteoclast function was first proposed in 1995 by Bowler et al. [156] after ATP was found to stimulate resorption by cells from human osteoclastoma. Originally, this effect was proposed to be mediated via the P2Y₂ receptor; however, in a follow-up study, UTP failed to stimulate resorption suggesting this was not the case [259]. Subsequently, ATP was found to stimulate the formation and activity of rodent osteoclasts, effects which were inhibited by apyrase or suramin [12]. These stimulatory actions on resorption were further increased when osteoclasts were first activated by culture in acidified medium [12]. Since the P2X2 receptor is the only P2 receptor subtype that requires extracellular acidification to show its full sensitivity to ATP [260], it was suggested that these pro-resorptive effects could involve the P2X2 receptor. Further investigation showed that low micromolar concentrations of ADP, 2-meththio ADP and ATP potently stimulated osteoclast formation and activity [261]. Pharmacological and cytochemical evidence suggested that these effects were mediated via P2Y₁ receptors [261]. Analysis of skeletal changes in P2Y₁ receptor knockout mice by DEXA and μCT showed reduced trabecular bone in the long bones [117, 118]. This decrease in bone mass was unexpected given the previously suggested role for the P2Y₁ receptor in osteoclast activity. However, a recent study found that ADP still stimulated osteoclast formation and resorption in cells derived from P2Y₁ receptor knockout mice [120], suggesting the involvement of another P2Y receptor. Based on pharmacology, the logical alternative candidate for mediating the

effects of ADP on osteoclasts was the P2Y₁₂ receptor. The P2Y₁₂ receptor was only identified in 2001 [262], which was the same year as the initial study by Hoebertz et al. and thus was not considered in the original study. Furthermore, expression of the P2Y₁₂ receptor by osteoclasts has only recently been reported [119]. There is now evidence to suggest that the P2Y₁₂ receptor plays a key role in mediating the effects of ADP on osteoclast formation and activity since cells derived from P2Y₁₂ knockout mice display impaired responses to ADP [120]. Consistent with effects mediating osteoclast activity, P2Y₁₂ receptor knockout mice have increased trabecular bone and decreased arthritis associated bone loss [120]. Furthermore, clopidogrel, a P2Y₁₂ receptor antagonist, inhibits osteoclast formation, viability and resorptive activity [168].

It has also been suggested that ATP stimulates human osteoclast activity indirectly through upregulation of osteoblast-expressed RANKL [258], and that these effects are probably mediated via P2X7 receptors [263]. Interestingly, osteoclasts isolated from human deciduous teeth respond to ATP by activating K^+ and Cl^- currents, and this may also contribute to the stimulatory effects of ATP on bone resorption [264].

It is now emerging that the P2Y₆ receptor may play an important role in osteoclasts. The P2Y₆ receptor has been implicated in osteoclast survival since activation by its agonist, uridine diphosphate (UDP), prevented the apoptosis induced by tumour necrosis factor- α (TNF- α) [265]. UDP also induced the translocation and activation of NF- κB in osteoclasts and their precursors [265]. Recently, activation of the P2Y₆ receptor by UDP has been shown to stimulate osteoclast formation and activity [119]. Consistent with a role for this receptor in bone resorption, osteoclasts derived from P2Y₆ receptor knockout mice displayed defective function [119]. Analysis of P2Y₆ receptor knockout mice by μCT revealed increased cortical bone in the long bones and spine [119].

The effect of P2Y₁₃ receptor activation on osteoclast function requires further investigation. However, P2Y₁₃ receptor knockout mice display reduced osteoclast numbers in vivo with protection from ovariectomy-induced bone loss in females [121].

The role of the P2X7 receptor on osteoclast formation and activity appears complex. An initial study showed that P2X7 receptors enhanced giant cell formation [128]. High concentrations of ATP were also reported to activate P2X7 receptors on rabbit osteoclasts, and it was suggested that ATP released in response to mechanical stimuli may act via P2X7 receptors to inhibit osteoclastic resorption [266]. Experiments using cells derived from human peripheral blood showed that antagonism of the P2X7 receptor with oxidised ATP or a monoclonal antibody inhibited the fusion of osteoclast precursors to form multinucleated osteoclasts [267]. Analogues of the P2X7 receptor antagonist, KN-62, have also been shown to induce osteoclast apoptosis [268]. In contrast, Ke et al. demonstrated that P2X7 receptor knockout mice possessed functional osteoclasts in vivo [137]. In addition, using knockout precursor cells, osteoclasts

were generated *in vitro*, suggesting that the P2X7 receptor is not required for cell fusion [137]. Other studies have also shown that P2X7 receptor knockout mice retain the ability to form multinucleated cells both *in vitro* and *in vivo* [114, 116]. RAW 264.7 osteoclast-like cells display P2X7 pore-forming activity and prolonged exposure to ATP was found to down-regulate P2X7 receptor function and inhibit osteoclast formation [269].

Activation of the P2X7 receptor has also been shown to induce the translocation of NF- κ B [263] and an isoform-specific PKC in osteoclasts and their precursors [270]. The P2X7 receptor may additionally play a role in the intercellular communication between osteoblasts and osteoclasts [271], cytoskeletal reorganisation at the sealing zone and the delivery and secretion of lytic granules into the resorption lacunae [272]. Recently, it was shown that ATP release from osteoclasts is mediated via the P2X7 receptor [211, 273]. Thus, this receptor is thought to drive osteoclast fusion by increasing the concentration of extracellular adenosine (via breakdown of released ATP) [273] (see Fig. 3).

How stimulation of the P2X1, P2X2, P2X3, P2X4, P2X5, P2Y₂ and P2Y₁₄ receptors influences osteoclast formation and activity remains to be established; however, a number of other effects of extracellular ATP on osteoclasts have been reported. Osteoclasts secrete L-glutamate upon stimulation by ATP [274] and ATP released from osteoclasts is thought to sensitise bone to the action of parathyroid hormone and other systemic hormones [275]. Chemical sympathectomy was found to impair bone resorption in rats [276], perhaps mediated by ATP released as a cotransmitter acting on osteoclasts. Furthermore, ATP flowing from cells after surgery can directly activate P2X4 receptors on the sensor cells of marginal gingiva or by direct activation of osteoclasts on the bone surface [277]. A recent study suggested that intracellular ATP levels play a pivotal role, not only in osteoclast apoptosis, but also in the bone-resorbing function of the cells [278]. Bisphosphonates are highly effective inhibitors of bone resorption via a

mechanism involving a novel ATP analogue (1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl)) ester triphosphoric acid (Apppl) that leads to the accumulation of isopentenyl pyrophosphate; Apppl then induces osteoblast apoptosis [279].

P1 receptors, adenosine and osteoclasts

A potential role for adenosine in regulating osteoclast function was first suggested in the 1980s when 2-chloroadenosine, an agonist at P1 receptors, was shown to stimulate bone resorption in calvarial bones in organ culture; however, the underlying mechanism was not clear [280]. It was later proposed that PGE₂ and 2-chloroadenosine act in concert to stimulate bone resorption [281]. Adenosine produced after hydrolysis of ATP released from osteoprogenitor and BMS cells acts on P1 receptors leading to the release of IL-6 and inhibition of osteoprotegerin secretion, which results in the stimulation of osteoclastogenesis [244]. It has been shown that endogenous adenosine acting via A₁ receptors on osteoclasts and their precursor cells plays a critical role in regulating bone turnover [282]. A₁ receptor antagonists regulate osteoclastogenesis by bone marrow osteoclast precursor cells by inhibiting RANKL-induced activation of the JNK/c-Jun pathway [283]. Consistent with these findings, A₁ receptor knockout mice display increased bone mineral density and resistance to ovariectomy-induced bone loss [284]. ATP released through P2X7 receptors acts as the major source of adenosine that enhances mature osteoclast function via A_{2A} receptors [273]. However, a later study showed that adenosine, acting through A_{2A} receptors, inhibited osteoclast differentiation [285]. Thus, the authors suggested that adenosine may regulate bone turnover under conditions when levels are elevated [285]. A_{2A} receptor activation has also been shown to prevent the osteolysis that occurs during prosthesis loosening with particle-induced inflammation, a common cause of joint implant failure [286]. In addition, adenosine blocks aminopterin-induced suppression of osteoclast differentiation and osteoclastogenesis [287].

Bone marrow stromal cells or mesenchymal stem cells

A key function of bone is to protect the bone marrow, the site of blood cell formation. Osteoblasts and lining cells (or quiescent osteoblasts) located on the bone surface form a continuum with stromal cells of bone marrow. It has been reported that osteoclasts promote the formation of hematopoietic stem cell niches in bone marrow [288]. Osteoblasts, adipocytes and chondrocytes are all derived from mesenchymal stem cells (MSCs) and thus these cells play a key role in bone. The number of investigations examining the role of purinergic signalling in MSCs is limited; however, an important role for extracellular nucleotides in regulating MSC proliferation and differentiation is emerging. Initial studies demonstrated that fluid flow induced vesicular release of ATP from

Fig. 3 Schematic diagram showing release of ATP from osteoclasts via P2X7 receptors, where it undergoes enzymatic breakdown to AMP (via apyrase) and adenosine (Ado; via CD73). Ado acts on P1 (A₁ and/or A_{2A}) receptors to stimulate osteoclast fusion

human MSCs, which then stimulated their proliferation via purinoceptor-mediated increase in $[Ca^{2+}]_i$ [289]. UTP and, to a lesser extent, ATP were also shown to stimulate human MSC migration [290]. Human MSCs express all P2Y receptors and the P2X3–7 receptors [291, 292]. Recently, extracellular nucleotides have been reported to modulate the differentiation of human MSCs, with the P2X6, P2Y₄ and P2Y₁₄ receptors identified as playing pivotal roles in this process [292, 293]. UDP activation of P2Y₆ receptors has also been shown to regulate the osteogenic differentiation of the primary MSCs from postmenopausal women [291]. The same study also demonstrated that NTPDases influence which osteoblast progenitors are driven into proliferation or differentiation [291]. Furthermore, a detailed investigation using knockout mice identified that the P2Y₁₃ receptor regulates the terminal differentiation of MSCs into osteoblasts or adipocytes [207].

In terms of P1 receptor signalling, many of the studies investigating the role of adenosine in osteoblasts have utilised MSCs (see above). However, activation of A₁, A_{2A}, A_{2B} and A₃ receptors has been shown to increase the proliferation (mainly via A₁ and A_{2A} receptors) and differentiation (mainly via A_{2B} receptors) of MSCs [245]. Adenosine also inhibits chemotaxis and induces hepatocyte-specific genes in MSCs [294].

Cartilage and chondrocytes

Cartilage is a flexible connective tissue found in many areas of the body, in the skeletal system it is particularly important in the joints. The extracellular matrix of cartilage is composed of collagen, abundant ground substance rich in proteoglycans and elastin fibres. This matrix is produced by chondroblasts, which, when they become embedded within the extracellular matrix in lacunae, are called chondrocytes. Cartilage does not contain blood vessels since chondrocytes produce an anti-angiogenesis factor that discourages their formation.

P2 receptor expression by chondrocytes

Purinergic receptor expression in cartilage was first suggested in the early 1990s when ATP was shown to stimulate resorption of bovine nasal cartilage, an effect that was not mediated by adenosine or prostaglandins [295]. The following year, ATP was shown to stimulate PGE₂ production from articular chondrocytes [296]. P2 receptors were later identified on human articular chondrocytes that were responsive to UTP as well as ATP [297]. Subsequently, P2U (P2Y₂ and/or P2Y₄) receptors were identified on sheep chondrocytes [298, 299]. Chondrocytes have now been shown to express many P2 receptors including the P2X1, P2X2, P2X3, P2X4, P2X7, P2Y₁ and P2Y₂ receptors [300–303]. Some of these receptors (P2X2, P2X4, P2X7 and P2Y₁) were expressed throughout all the zones of articular cartilage, whilst the P2Y₂ receptor

showed more limited expression only being present on superficial zone cells [302].

The presence of functional P2 receptors in chondrocytes has been demonstrated by a number of studies looking at intracellular Ca^{2+} levels. Early work showed that ATP and ADP induced a rise in $[Ca^{2+}]_i$ in growth plate chondrocytes [304], probably via P2Y receptors, since the calcium was released from intracellular stores. ATP also elevated $[Ca^{2+}]_i$ in retinoic acid-treated embryonic chondrocytes [305] and induced increases in $[Ca^{2+}]_i$ in human chondrons (chondrocytes surrounded by their native pericellular matrix and cultured in three-dimensional agarose films) [306]. In addition, UTP caused a transient increase in $[Ca^{2+}]_i$ and the release of ATP from cultured chondrocytes, effects that were reduced by suramin and blockers of Cl^- channels [307].

Functional effects of P2 receptor-mediated signalling in chondrocytes

Early work suggested that ATP caused articular cartilage mineralisation by promoting matrix vesicle-mediated calcium deposition [308, 309]. A specific ATPase, rather than non-specific alkaline phosphatase, was claimed to mediate this ATP-initiated calcification by matrix vesicles [310].

Recently, many of the studies describing the effects of purinergic signalling on cartilage have been conflicting with some reports suggesting that extracellular nucleotides negatively regulate cartilage metabolism whilst others describe beneficial effects of ATP. Activation of the P2Y₁ receptor by ATP inhibited cartilage formation in micromass cultures of chick limb undifferentiated mesenchyme cells [311]. ATP was also reported to promote proteoglycan breakdown and glycosaminoglycan release in bovine nasal cartilage [312] and increase the production of inflammatory mediators, nitric oxide (NO) and PGE₂ [303]. Furthermore, in articular chondrocytes, stimulation of P2Y₂ receptors by ATP increased IL-1-mediated PGE₂ release [300] and induced a rapid rise in PGE₂ synthesis via an ERK1/2 and p38 MAPK-mediated activation of cytoplasmic PLA₂ [313]. P2Y receptor signalling through MAPK/ERK was dependent on β 3 integrin ligation and was therefore influenced by the composition of the extracellular matrix [301]. In contrast, extracellular ATP and UTP stimulated cartilage proteoglycan and collagen accumulation in bovine chondrocyte pellet cultures [314, 315]. ATP, released by compression, also suppressed the release of NO and stimulated proteoglycan synthesis in chondrocytes/agarose constructs [316, 317].

The response of human articular chondrocytes to ATP is enhanced by IL-1 β [297] and TNF- α [318, 319]. Activation of P2Y receptors also leads to elevated fibroblast growth factor-induced proliferation [298, 299]. In addition, ATP signalling via the P2X4 receptor is thought to mediate the increased $[Ca^{2+}]_i$ required for chondrocyte differentiation [320].

A recent study has also shown that intracellular oscillations in ATP have a critical role in pre-chondrogenic condensation, via P2X4 receptors [321] and mediate inductive action of fibroblast growth factor [322].

The repair of foetal, but not adult, articular cartilage involves ATP-induced increases in $[Ca^{2+}]_i$ and expression of *c-fos* [323]. Thus, harnessing the ATP receptor pathway has been shown to be a highly effective approach to improve tissue formation and impart functional mechanical properties of engineered cartilage constructs used to repair damaged articular cartilage [324].

ATP release and breakdown in cartilage

The first direct evidence for ATP release from chondrocytes came in 2000 from a study by Graff et al. [325]. They showed that ATP was continuously released from chondrocytes at rest and in response to mechanical load to promote matrix mineralisation [325, 326]. Earlier, hypotonically induced stretch had been demonstrated to increase ion channel activity in cultured human articular chondrocytes [327], an effect which, in retrospect, was probably mediated via ATP release. Similarly, the increased $[Ca^{2+}]_i$ which is observed in articular chondrocytes following fluid flow [328] and pressure-induced strain [329] is probably due to the release of ATP. Additionally, ATP stimulation of P2Y receptors elicits an increase in intracellular $[Ca^{2+}]_i$ which triggers further release of ATP from adjacent cells, thereby enhancing Ca^{2+} waves in chondrocytes [307]. There has been a recent report that 100 μ M ATP induced massive release of ATP from articular chondrocytes, probably as the result of P2X7 receptor activation [330].

Physiological joint loading and the associated compression of articular cartilage produce events that influence both chondrocyte metabolism and extracellular matrix synthesis. It has been shown recently that primary cilia are essential for chondrocyte mechanotransduction and the control of extracellular matrix secretion; in particular, chondrocyte primary cilia are required for ATP release and purinoceptor-mediated Ca^{2+} signalling [331]. ATP transport from chondrocyte primary cilium was, at least in part, via connexin 43 hemichannels [302, 332]. Release of ATP via pannexin 3 hemichannels has also been proposed [333]. The authors suggested that pannexin 3 functioned to switch chondrocyte cell fate from proliferation to differentiation by regulating the intracellular ATP/cAMP levels.

ATPase activity in chondrocytes was first reported by in 1983 by Kanabe et al. [334]. It was later shown that the ecto-enzymes NTPDase, NPP, alkaline phosphatase and 5'-nucleotidase, as well as adenylate kinase and nucleoside diphosphokinase which mediate regeneration of ATP, are differentially localised in cartilage [326]. P1 receptor agonists decreased extracellular PP_i production surrounding chondrocytes and cartilage explants, while ADP increased PP_i levels,

suggesting a homeostatic role for P1 and P2 receptors in stabilizing concentrations of PP_i [335].

P1 receptors, adenosine and chondrocytes

The regulation of chondrocyte function by P1 receptors was first suggested following the observation that adenosine inhibited proliferation and produced damage to cultured chondrocytes from rabbits [336]. Later, it was demonstrated that chondrocytes respond to adenosine via A_2 receptors, activity which was potentiated by adenosine deaminase (ADA) and phosphodiesterase inhibitors [337]. Furthermore, pulsed electromagnetic fields produce an up-regulation of the A_{2A} and A_3 receptors that mediate proliferation of bovine chondrocytes and fibroblast-like synoviocytes [338], and A_{2A} receptor activation reduces inflammation in mouse articular chondrocytes stimulated with IL-1 β [339].

Ligaments and tendons

Ligaments

A role for purinergic signalling in ligaments has only recently emerged, and to date, most of the work has been performed on periodontal ligament cells. In these cells, ATP stimulates RANKL expression via a P2Y₁ receptor-cyclooxygenase-dependent pathway [340]. Mechanical stress causes ATP release, which acts via P2Y₁ receptors to increase expression of osteopontin, a protein involved in bone remodelling [341, 342]. Evidence suggests that the ATP release from periodontal ligament cells occurs via connexin 43 hemichannels [343]. Furthermore, RT-PCR analysis has shown that these cells express mRNA for P2X7, P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₂ receptors. Release of ATP from human periodontal ligament cells in response to mechanical stress also leads to IL-1 β expression via P2X7 receptor activation [344]. Combined, these studies indicate that extracellular ATP may play an important physiological role in periodontal remodelling.

Tendons

There is significant evidence to suggest an important role for extracellular ATP in tendons. Human tendon cells respond to mechanical stimulation by increasing $[Ca^{2+}]_i$. This effect was blocked by thapsigargin [345], suggesting that ATP was released and then activated P2Y receptors. Indeed, a later paper showed that mechanical loading induced ATP release from tendon cells and stimulated expression of IL-1 β , cyclooxygenase-2 and matrix metalloproteinases, triggering matrix remodelling or a destructive pathway [346]. The authors suggested that the released ATP may serve as a negative feedback mechanism to limit activation of the injurious

pathways. Mechanical loading also stimulated ecto-ATPase activity in human tendon cells and motion may release ATP from tendon cells in vivo [347]. Thus, it was suggested that the ATP/ATPase system may be important for tendon homeostasis by protecting tendon cells from responding to excessive load signals. P2 receptor blockade with pyridoxalphosphate-6-azophenyl-2',5'-disulfonic acid significantly attenuated the responses of group III myelinated afferent nerves to tendon stretch, contributing to the exercise pressor reflex [348], in keeping with the claim that mechanical stimulation releases ATP from tendon cells.

Diseases of the musculoskeletal system

Skeletal muscle diseases

ATP was used early in the therapy of myopathies ('Adotripos', Rona, UK), although the mechanism of action was unknown. In rat soleus muscles, the stimulatory effect of insulin on D-xylose uptake was progressively lost and was associated with the loss of muscle ATP; extracellular ATP promoted an effect of insulin in otherwise insulin-unresponsive muscles [349]. Exogenously added ATP was also shown to abolish the adverse actions of PLA₂, local anaesthetics and the Ca²⁺ ionophore A23187, as well as lithium, on carbohydrate metabolism in the isolated rat diaphragm muscle [350]. It was suggested that ATP may have therapeutic potential, as it may stimulate the impaired glycolysis induced by various drugs and conditions which cause muscle weakness or damage.

Adult injured muscles undergo regeneration and connexin hemichannels are re-expressed in undifferentiated cells that form new myofibres, supporting the healing process of injured muscle following mediation of the release of ATP [92].

Muscular dystrophy

Duchenne muscular dystrophy (DMD) is the most common and severe of muscular dystrophies. It is caused by mutations in the dystrophin gene resulting in loss of dystrophin protein. Allopurinol has been used for the treatment of DMD since it counteracts the reduction in purine nucleotide degradation levels in Duchenne muscle. However, it has been reported that chronic administration of allopurinol and adenine does not improve the clinical status of DMD [351]. Early work revealed that lymphoblastoid cells from DMD patients were highly sensitive to stimulation by extracellular ATP [352].

Some muscular dystrophies are caused by a sarcoglycan gene mutation. α -Sarcoglycan has been reported to have ecto-ATPase activity and it was suggested that the activity of P2X receptors may be modulated by α -sarcoglycan which buffers the extracellular ATP concentration [353]. Elevated concentrations of extracellular ATP and its activation of P2X

receptors lead to Ca²⁺ overload and muscle fibre death in the absence of α -sarcoglycan in sarcoglycanopathies.

The *mdx* model of DMD, which lacks dystrophin protein, has been used as a clinically relevant form of muscle damage and subsequent regeneration. Using a combination of immunohistochemistry, RT-PCR and electrophysiology, sequential expression of P2X₅, P2Y₁ and P2X₂ receptors was shown during the process of muscle regeneration in the *mdx* model [354]. The P2X₅ and P2Y₁ receptors were expressed first in activated satellite cells. The P2Y₁ receptor was also expressed on infiltrating immune cells. P2X₂ receptor expression on newly formed myotubes showed colocalization with ACh receptors, suggesting a role for P2X₂ receptors in the regulation of muscle innervation. Evidence for the involvement of purinergic signalling in muscle regeneration may lead to novel therapeutic strategies for the treatment of muscle disease. Immortalized myoblast cell lines derived from the *mdx* mouse (SC5) and control (dystrophin positive) myoblasts (IMO) from the same parent mouse strain have been used to investigate changes in P2X receptor expression and function in dystrophic muscle [355]. It was shown that a purinergic dystrophic phenotype arises at the earliest myoblast stage of developing dystrophic muscle. Dystrophic-negative myoblasts were more sensitive to ATP, resulting in increases in [Ca²⁺]_i. P2X₄ and P2X₇ receptor proteins were also expressed on dystrophic myoblasts. It was suggested that P2X₄ and P2X₇ receptor antagonists may be of potential therapeutic benefit.

Myofascial pain is common in DMD and ATP has been claimed to excite or sensitise myofascial nociceptors [356–358]. P2X₇ receptor upregulation was shown in dystrophic *mdx* mouse muscles and it was suggested that treatment with P2X₇ receptor antagonists might slow the progression of muscular dystrophy [359]. Sensitivity to ATP was reported to be much higher in *mdx* mice [360]. ATP release is greater in *mdx* mice and there is increased expression of P2Y₂ receptors, but a decrease in P2Y₁ receptors [361].

Myasthenia gravis

Myasthenia gravis is an autoimmune disease, affecting the neuromuscular junction. It has been reported that lack of neuronal A_{2A} receptor-mediated Ca_v 1 (L-type) influx causes tetanic failure in myasthenia gravis [362].

Fibromyalgia

Fibromyalgia is characterised by pain and allodynia, as well as mood disorders. It has been considered as a musculoskeletal disease, but also more recently as a neuropsychiatric condition. It has been shown that there are significantly lower levels of ATP in platelets of fibromyalgia patients and it was suggested that this may be of relevance in the pathogenesis of the disease [363].

Bone and joint disorders

Osteoporosis

Osteoporosis is a condition characterised by a loss of bone mass (decreased bone mineral density), leading to increased risk of bone fracture. It occurs most commonly in postmenopausal women. Of the P2 receptor subtypes expressed by bone cells, it is the P2X7 receptor which has received the most attention in relation to the treatment of osteoporosis. Initial work using animal models demonstrated that the P2X7 receptor plays an important role in both cortical and cancellous bone mass augmentation in rapidly growing female mice [364]. Furthermore, P2X7 receptors were shown to mediate stimulation of periosteal and cancellous bone formation and the inhibition of cancellous bone resorption during growth [115]. The P2X7 receptor is highly polymorphic and a number of studies have shown that single nucleotide polymorphisms (SNPs) of the P2X7 receptor gene are associated with fracture risk, decreased bone mineral density and osteoporosis (see [222–224, 365]). P2X7 receptors are also involved in the complex chain of events leading to the formation of human osteoclasts, and it was suggested that P2X7 receptor antagonists may be valuable for the management of osteoporosis and with disorders of remodelling, where there is reduced bone mass [366, 367].

Non-synonymous polymorphisms in the P2X4 receptor gene and the Leu46Pro polymorphism of the human P2Y₂ receptor gene have also been associated with bone mineral density and osteoporosis risk in a cohort of Dutch fracture patients [368]. Furthermore, it has been reported recently that clopidogrel, a P2Y₁₂ receptor antagonist widely used for thrombosis and stroke, is associated with risk of osteoporotic fractures [206].

Adenosine receptors have also been implicated in osteoporosis [369]. Studies have suggested that endogenously released adenosine, acting on A₁, A_{2A} and A_{2B} receptors on osteoclasts and their precursor cells, plays a critical role in regulating bone turnover [370, 371]. Furthermore, transdifferentiation of osteoblasts to adipocytes, a process which involves A_{2B} receptors, may also contribute to the pathogenesis of osteoporosis [372, 373]. Thus, P1 receptors may be useful targets in treating diseases characterised by excessive bone turnover, such as osteoporosis.

Periodontitis

The direct cause of tooth loss in periodontitis is excessive resorption of alveolar bone, which supports tooth roots. FR202126, an ATPase inhibitor, inhibited bone resorption in calvaria cultures, which, it was suggested, may offer a novel approach for the treatment of periodontitis [374].

Paget's disease

This disease is characterised by an increase in osteoclast number, resulting in an excess of bone resorption and high bone turnover. Bisphosphonates have been used to treat diseases with excessive bone resorption and high bone turnover, such as Paget's disease. They act by inducing apoptosis of osteoclasts. It has been suggested that nitrogen-containing bisphosphonates block mitochondrial ADP/ATP translocase, which is involved in the induction of apoptosis [375]. P2X7 antagonists are also being considered for the treatment of bone diseases where there is an increase in osteoclasts number such as Paget's disease [366].

Rickets

Rickets is a bone disorder caused by a vitamin D and phosphate deficiency. In rachitic rats, induced by the use of synthetic diets, there was a decrease in whole blood ATP and serum phosphorus. Subcutaneous injection of ATP or inorganic phosphate caused an increase in serum phosphorus and blood ATP levels, and it was suggested that the metabolic disturbances in rickets were due to alteration in ATP production [376].

Ossification of the posterior longitudinal ligament of the spine

This disease is characterised by ectopic bone formation in the spinal ligament, which compresses the spinal cord, resulting in serious neurological damage. It is a common disease in Japanese and other Asian populations and has been treated by spinal cord decompression surgery. Work has shown that extracellular concentrations of ATP in ossification of the posterior longitudinal ligament of the spine (OPLL) cell cultures are significantly higher than those in non-OPLL cultures and P2Y₁ receptors are highly expressed in OPLL cells, but only weakly expressed in non-OPLL cells [377]. Furthermore, ATP and mechanical stress raise the mRNA levels of alkaline phosphatase and osteopontin in OPLL cells. These effects were blocked by MRS2179, a selective P2Y₁ receptor antagonist. A later study from this group showed that P2Y₁ receptor over-expression in OPLL-induced mineralisation leading to ectopic bone formation in the spinal ligament cell of OPLL patients [378].

Rheumatoid arthritis

RA is a disease of the synovial lining of joints. It is a complex, multi-factorial autoimmune disease and many patients show the presence of rheumatoid factor in their serum. The joints are initially painful, swollen and stiff, but as the disease progresses, the ligaments supporting the joints are damaged and there is erosion of the bone leading to deformity of the joints. Tendon sheaths can also be affected leading to tendon rupture.

The potential involvement of purinergic signalling in RA was first identified in the 1990s when adenosine concentrations, formed following the breakdown of released ATP, were lower in RA synovial fluid than in osteoarthritis (OA). Thus, the augmentation of adenosine levels was suggested as a treatment of this condition [379]. Compared to the joints from RA patients, higher levels of ATP and 5'-nucleotidase activity were also observed in the synovial fluid from osteoarthritic joints; especially from osteoarthritic patients in whom deposition of calcium-containing crystals was also present [380, 381]. Later, ATP and UTP were claimed to activate calcium-mobilizing P2U receptors to act synergistically with IL-1 to stimulate PGE₂ release from human rheumatoid synovial cells [382].

IL-1 β is a proinflammatory cytokine that is thought to play an important role in the progression of RA. ATP, acting via P2X7 receptors, induced significantly higher levels of IL-1 β in blood samples from RA patients compared to controls [383]. They suggested that mononuclear cells from these patients were more sensitive to ATP stimulation than those from healthy individuals, perhaps due to genetic polymorphism in the P2X7 gene. A separate study also reported increased IL-1 β release in RA patients and these investigators suggested that P2X7 receptors play a role in the pathogenesis of RA and systemic lupus erythematosus [384]. A recent paper has also shown that the His155Tyr (489C>T) SNP of the P2X7 receptor gene confers an enhanced function of P2X7 receptors in immune cells from patients with RA [385]. Human rheumatoid synoviocytes express P2X7 receptor mRNA and protein; stimulation of which leads to release of the cytokine IL-6, but does not cause pore formation and cell death [386]. The activating protein-1, particularly FosB, appears to be linked to P2X7 receptors in the control of immune and osteogenic function [387]. In addition, animal models of arthritis have provided evidence that suggests an *in vivo* role for the P2X7 receptor in the progression of inflammatory disease; P2X7 receptor knockout mice exhibited a reduced incidence and severity of anti-collagen-induced arthritis symptoms [388]. Thus, targeting the P2X7 receptor could be a promising treatment for RA [172]. Block of P2X7 receptor signalling in the collagen-induced arthritis animal model of RA inhibited peripheral inflammatory tissue destruction and the autoreactive humoral response, supporting the view that P2X7 receptor antagonists may cure inflammatory autoimmune diseases, such as RAs [389]. However, a later study claimed that AZD9056, a P2X7 receptor antagonist, did not appear to be a therapeutically useful target for RA [390]. P2X7 receptor antagonists are also being explored for the treatment of inflammatory pain in joints [391]. A multicentre, double-blind, placebo-controlled clinical trial was launched to see if ATP infusion therapy was effective in patients with RA [392], and later it was claimed that ATP infusions reduced inflammation and disease activity of a patient with RA [393].

Bovine chondrocytes express P2X1 and P2X3 receptors, which, following stimulation, lead to release of the inflammatory mediators NO and PGE₂ [303]. Thus, antagonists for these receptors may be potential targets for pathophysiological diseases linked to inflammation and articular cartilage resorption. Patients with RA have increased risk of cardiovascular disorders, and it was shown that there was an enhanced response of platelets to ADP in patients with RA [394].

Sympathetic nerves have proinflammatory effects during the early phase of type II collagen-induced arthritis, probably mediated by cytokines, such as interferon- γ released by cells in response to sympathetic cotransmitters [395], and perhaps involving ATP. An important study supported the notion that α , β -meATP-sensitive P2X (probably P2X3) receptors are present in peripheral nociceptive afferent fibres in the rat knee joint and ATP, levels of which are increased in inflamed and damaged tissues, may be involved in the initiation of nociception and pain [396]. P2X3 receptors were also described on nociceptive sensory fibres in lumbar facet joints, from which low back pain can originate [397]. It was also observed that changes in the expression of P2X3 receptors on isolectin B4-labelled DRG neurons occur after induction of RA [398]. In addition, it has been suggested that P2X3 receptors expressed on trigeminal ganglia play an important role in orofacial pressure pain caused by monoarthritis of the temporomandibular joint [399].

It has been postulated that the nervous system may play a role in the pathogenesis of rheumatic diseases, involving lymphocytes that are recognised to be innervated (see [400]). ATP, released as a cotransmitter from sympathetic nerves, enhanced bradykinin-induced plasma extravasation in the rat knee joint [401]. Intravenous guanethidine, that prevents the release of sympathetic cotransmitters, was shown to be effective in patients with RA [402] and it is possible that this is due to the pro-inflammatory effects of ATP, released as a cotransmitter from sympathetic nerves.

There is abundant evidence demonstrating a role for adenosine in the pathogenesis of RA (see review by Varani et al. [148]). Synovial fluid taken from patients with RA, but not OA, shows increased activity of ADA [403, 404]. An increase in ADA activity was also found in rheumatoid synovial fibroblasts [405]. Consequently, it was suggested that serum ADA may predict disease activity in RA [406]. It has been shown that the ImmunoKnow assay may be effective in identifying RA patients at increased risk of infection [407].

2-Chloroadenosine was shown to induce apoptotic cell death of fibroblast-like synoviocytes derived from patients with RA [408]. It was also reported that signalling via the A_{2A} receptor caused modification of the cytokine milieu in RA [409]. Release of TNF- α by adenosine was also substantially lower in patients with RA compared to control subjects [410]. However, in another study, the symptoms of RA were

not modified by treatment with the adenosine uptake inhibitor, dipyrindamole [411].

Overexpression of A₃ receptors was found in peripheral blood mononuclear cells of RA patients; upregulation was induced by inflammatory cytokines controlling the expression of the A₃ receptor transcription factor NF- κ B [412]. A₃ receptors were also found to be expressed at higher levels in the synovium of patients with RA [413]. Increased levels of A_{2A} and A₃ receptors on the lymphocytes and neutrophils of RA patients were also reported, and it was shown that anti-TNF- α normalized A_{2A} and A₃ receptor expression and functionality [414].

Adjuvant-induced monoarthritis is regarded as a useful model for RA and other chronic inflammatory conditions. Spinal cord adenosine receptor activation inhibits inflammation and joint destruction in rat adjuvant-induced arthritis [415]. In a later paper from this group, they showed that A₁ receptor agonists reduced *c-fos* and astrocyte activation in dorsal horn of rats with induced RA [416]. Later, an A₃-specific agonist was shown to prevent bone resorption in this model [417]. ATP, perhaps working via A₂ receptors, also substantially reduces joint injury [418]. The A₃ receptor agonist, CF502, reduced expression levels of protein kinases in fibroblast-like synoviocytes from RA patients and in adjuvant-induced arthritic rats [419]. Furthermore, block of the nucleoside transporter inhibited disease progression in rat RA models [420].

An early study reported the suppression of zymosan-induced inflammatory arthritis by intraperitoneal administration of adenosine [421]. Later evidence from a phase II clinical trial has suggested that A₃ receptor agonists could be used to treat RA [422–424]. Additionally, an agonist of the A_{2A} receptor, CGS 21680, reduced progression of murine type II collagen-induced arthritis [425] and activation of A_{2A} receptors on immune cells by a CD73-dependent prodrug decreased joint inflammation in experimental RA [426]. A phosphorylated A_{2A} receptor agonist, Chet-AMP, has been shown to have anti-inflammatory activity in collagen-induced arthritis, by acting locally on immune cells invading inflamed tissue [427]. A recent study has examined how effective A_{2A} receptor agonists are for the treatment of RA in relation to different pharmacological treatments and shown that they ameliorate adjuvant-induced arthritis in rats [428].

Methotrexate (MTX) is widely used for the treatment of RA. In human inflammatory joint disease, the modulation of orphan nuclear receptor expression by MTX involved A_{2A} receptor-mediated responses [429]. Furthermore, MTX-induced suppression of osteoclastic bone destruction in adjuvant-induced arthritis was abolished by extracellular adenosine, acting via A_{2B} receptors [430]. Gene–gene interactions in adenosine biosynthesis pathways have been shown to affect MTX efficacy and tolerability in RA [431]. Studies of polymorphisms in genes contributing to adenosine release in

RA patients treated with MTX led to the conclusion that genotyping may help in the identification of patients who would benefit most from MTX treatment [432].

Anti-TNF- α is also used as a treatment for RA, but it increases the risk of tuberculosis (TB) reactivation. Serum ADA levels increase in TB patients, and it was suggested that elevated ADA levels could be used for diagnosis of TB in patients being treated with anti-TNF- α [433]. Differences between ADA levels in joint effusion and serum of patients with RA and OA have been observed, and it was concluded that ADA assay could be a sensitive and specific test for the rapid diagnosis of rheumatoid effusions [434].

Osteoarthritis

OA is a degenerative disease of the joints resulting from wear of the articular cartilage, which leads to (or may even result from) secondary changes in the underlying subchondral bone. OA can be primary or secondary to abnormal load to the joint or from trauma, infection or ageing. It can be recognised by loss of cartilage and the presence of osteophytes, osteosclerosis and cysts in the bone. The pain from OA appears to be due largely to inflammation.

At present, information regarding the role of P2 receptor signalling in the pathogenesis of OA is limited. In guinea pig knee OA, a heightened generation of NO is related to the depletion of ATP in chondrocytes [435]. It was suggested that the elevation of the ecto-nucleotidase NPPI and its product PP_i, which directly stimulates chondrocalcinosis, may be driven by chondrocyte ATP depletion.

ATP has been shown to directly contribute to pathologic mineralisation in articular cartilage, and thus, P2 receptor antagonists may lead to effective therapies for crystal-associated arthritis [436]. Levels of ATP in the synovial fluid are also related to the pain intensity in patients with OA of the knee [437]. Different polymorphic variants of the P2X₇ receptor have been associated with high or reduced periprosthetic osteolysis in the long-term complication of the total hip arthroplasty [438].

Adenosine signalling also appears to be involved in OA. Adenosine excites nociceptive afferents of the rat knee joint via A₁ receptors [439]. A study using the murine osteoarthritic STR/ort strain showed that increased adenosine production may induce chondrocyte apoptosis and play a role in OA in the tibial cartilage [440]. Adenosine, produced after breakdown of ATP released from chondrocytes, plays a role in the response to tissue damage in arthritic conditions and inhibition of adenosine kinase attenuates IL-1- and lipopolysaccharide (LPS)-induced changes in articular cartilage in arthritis [441]. In addition, A_{2A} receptors are involved in the regulation of LPS-induced IL-6 release, implying a regulatory role for adenosine receptors in the control of inflammation and osteoclastogenesis [442].

Synovial fibroblasts can contribute to the development of OA by the secretion of pro-inflammatory mediators. Electromagnetic field stimulation led to up-regulation of A_{2A} receptors in synovial fibroblasts and it was suggested that adenosine, acting through both A_1 and A_{2A} receptors, had anti-inflammatory activity to control joint inflammation [443]. A_{2A} and A_3 receptor agonists have been shown to modulate PGE_2 and cytokine release in human osteoarthritic fibroblasts [444]. Adenosine receptors also regulate inflammatory responses in human synoviocytes [445].

A_{2A} receptor agonists have been used to reduce joint destruction due to septic arthritis [446] and they may reduce the destructive effects of polyethylene wear debris and prevent joint prosthesis loosening [447].

Bone cancer and multiple myeloma

Bone metastases, the most common type of metastases for patients with breast and prostate cancer, can result in significant bone loss, fractures, pain, hypercalcemia and spinal cord compression. Skeletal metastases have been radiographically classified as osteoblastic or osteolytic. Osteoblastic lesions are characteristic of prostate cancer, while in breast cancer, osteolytic lesions are found in 80 % of patients with stage IV metastatic disease. Complex cross-talk between tumour cells and the bone microenvironment promotes an active cycle of bone metastases through multiple extracellular factors, probably also including nucleotides (see [448, 449]).

The involvement of purinergic signalling in bone cancer was first noted in the 1990s when P2U (i.e. $P2Y_2/P2Y_4$) receptors were cloned and sequenced from osteoclastoma, a rare bone tumour caused by a malignant proliferation of osteoclasts [156]. The expression of $P2Y_2$ receptor transcripts from human osteoclasts from giant cell tumour of bone was subsequently reported [259]. Butyl benzyl phthalate, which is an endocrine disruptor that is known to interfere with mammalian ion channel receptors, suppressed ATP-induced cell proliferation mediated by P2X receptors in human osteosarcoma HOS cells [450]. Early work also highlighted that ATP was useful for purging residual tumour cells in autologous bone marrow transplantation [451]. Furthermore, APT102, a novel form of human apyrase/ADPase, works together with aspirin to disrupt bone metastasis in mice [452]. It is conceivable that ATP released from tumour cells could further stimulate osteoclast formation and activity [12], thus contributing to the so-called vicious cycle of bone destruction that commonly occurs around tumour metastases.

Bisphosphonates, drugs routinely used to treat osteoporosis, have been used for the treatment of bone cancer. The anti-tumour effects of these drugs are not clear but could involve apoptosis induced by the ATP analogue ApppI produced by nitrogen-containing bisphosphonates [453, 454].

There is compelling evidence that purinergic signalling is involved in cancer pain. Radiation therapy has been used to relieve bone pain and it was suggested that it may be related to the Ca^{2+} -signalling cascade, mediated at least in part by P2X6 receptors [455]. Experiments using a murine model of cancer pain demonstrated increased expression of P2X3 receptors on calcitonin gene-related peptide immunoreactive epidermal nerve fibres during tumour growth, suggesting a role for ATP in cancer-related pain [456]. In an impressive study, systemic blockade of P2X3 and P2X2/3 receptors with AF-353 was shown to substantially attenuate bone cancer pain behaviour in rats [457]. Chronic administration of the selective P2X3 and P2X2/3 receptor antagonist, A-317491, was also shown to transiently attenuate cancer-induced bone pain in mice in the early stages, but not in the later stages [458]. Perhaps the differences between the two stages can be explained by the fact that A-317491 has high plasma protein binding and limited CNS penetration, while AF-353 has low plasma protein binding and good CNS penetration, suggesting that there may be a central component of action against bone cancer pain. Functional up-regulation of P2X3 receptors has also been described in DRG in a rat model of bone cancer pain [459].

P2X7 receptor knockout mice are susceptible to bone cancer pain and had an earlier onset of pain-related behaviours compared to cancer-bearing, wild-type mice [460]. P2X7 receptor activation also induces cell death and cell surface CD23 (the low affinity IgE receptor) shedding from lymphocytes in human RPMI 8226 multiple myeloma cells [461]. A recent paper has presented evidence that $P2Y_1$ receptor signalling in the spinal cord and DRG may mediate bone cancer pain through the ERK pathway [462].

Multiple myeloma is a cancer of plasma cells but is often characterised by osteolytic bone lesions, primarily caused by increased osteoclast activity. It has been suggested that A_2 receptors may be useful targets for the treatment and prevention of multiple myeloma-induced bone disease since activation of A_{2A} receptors diminishes osteoclast function, while activation of A_{2B} receptors promotes osteoblast differentiation [463].

8-Amino-adenosine has also been proposed as a potential therapeutic agent for the treatment of multiple myeloma [464].

Severe combined immunodeficiency (SCID)

Genetic defects in the ADA gene are a major cause of SCID. Lack of ADA causes accumulation of adenosine. About a half of early-onset ADA-deficient patients exhibited bone defects due to decreased osteoclastogenesis and an intrinsic defect of osteoblasts function with subsequent low bone formation [465]. Additionally, they showed that the bone marrow microenvironment of ADA-deficient mice displayed a reduced capacity to support hematopoiesis and that treatment of ADA-deficient mice with enzyme replacement, gene therapy or

bone marrow transplantation resulted in full recovery. Gene therapy with ADA-transduced hematopoietic stem cells also improved children's growth.

Desbuquois dysplasia

This is an autosomal-recessive chondrodysplasia, characterised by severe prenatal and postnatal growth retardation, joint laxity, short extremities and progressive scoliosis. Calcium-activated nucleotidase 1 gene mutations have been identified in nine families with Desbuquois type 1, a gene that hydrolyses UDP followed by guanosine diphosphate and UTP [466].

Dwarfism (achondroplasia)

This is a congenital skeletal dysplasia due to a mutation in the gene that encodes the fibroblast growth factor receptor type 3, which acts through the MAPK cascade. Diadenosine tetraphosphate, Ap₄A, diminished the expression of the achondroplastic FGFR3 receptor, stimulating FGFR3 receptor degradation [467]. In a later paper from this group, it was shown that P2Y₁, P2Y₂, P2Y₆ and P2Y₁₁ receptors were expressed by achondroplastic chondrocytes and that Ap₄A was acting via these P2Y receptors [468]. In a more recent study, they showed that Ap₄A reversed the morphological changes triggered by FGF9 and restored the chloride efflux, providing further evidence for the therapeutic potential of this dinucleotide in the treatment of dwarfism [469].

Concluding remarks

Current evidence shows that purinergic signalling exerts complex local effects on the function of musculoskeletal cells. The effects of this signalling system are influenced by a number of factors including the receptor subtype, the extracellular nucleotides present locally and the expression of ecto-nucleotidases. In some cases, there appears to be a degree of redundancy present with several receptor subtypes mediating similar functional effects. Purinergic signalling has now been implicated in many disorders associated with the musculoskeletal system and thus presents many potential targets for future therapies. It should be noted that, given the ubiquitous nature of these receptors, selectively targeting specific cell types may prove challenging. In this respect, targeting the ecto-nucleotidases responsible for extracellular nucleotide breakdown or ATP transport inhibitors could also prove beneficial.

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