Pyrophosphate: a key inhibitor of mineralisation

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Abstract

Inorganic pyrophosphate has long been known as a by-product of many intracellular biosynthetic reactions, and was first identified as a key endogenous inhibitor of biomineralisation in the 1960s. The major source of pyrophosphate appears to be extracellular ATP, which is released from cells in a controlled manner. Once released, ATP can be rapidly hydrolysed by ecto-nucleotide pyrophosphatase/phosphodiesterases to produce pyrophosphate. The main action of pyrophosphate is to directly inhibit hydroxyapatite formation thereby acting as a physiological “water-softener”. Evidence suggests pyrophosphate may also act as a signalling molecule to influence gene expression and regulate its own production and breakdown. This review will summarise our current understanding of pyrophosphate metabolism and how it regulates bone mineralisation and prevents harmful soft tissue calcification.

Introduction

Inorganic pyrophosphate (or PP_i) is so named because it was originally prepared by heating phosphates (*pyro* from the Greek meaning "fire"). It comprises two inorganic phosphate (or P_i) molecules joined by a hydrolysable ester bond (**Figure 1**). Although pyrophosphate and longer chain polyphosphates can be synthesised under some circumstances, particularly by bacteria and non-mammalian organisms, pyrophosphate is not thought to be produced directly by mammalian cells. Instead, it is mainly generated by the hydrolysis of the phosphodiester bond in nucleotide triphosphates such as ATP or UTP. As such it is a metabolic by-product for many intracellular biochemical reactions and extracellular signalling cascades. The biology and biochemistry of pyrophosphate in nature has been expertly and comprehensively reviewed in a monograph by Heinonen published in 2001 [1].

It is important to distinguish between the roles of intracellular pyrophosphate, produced as a by-product of over 200 different enzyme reactions, and extracellular pyrophosphate which is separately regulated. In the 1950s, Kornberg and colleagues recognised that the hydrolysis of intracellular pyrophosphate was a major mechanism for driving biosynthetic reactions in the direction of synthesis [2]. Huge amounts of pyrophosphate are produced within cells daily, particularly during the generation of macromolecules such as proteins, nucleic acids, carbohydrates and lipids from their smaller precursors. For example, it has been estimated that ≥30g of pyrophosphate is generated daily by albumin synthesis within the adult human liver [3]. Clearly most of this pyrophosphate remains within cells where it is hydrolysed by intracellular pyrophosphatases.

Extracellular pyrophosphate: the "early years"

Pyrophosphate and polyphosphates are good complexing agents for metal ions (e.g. calcium and transition metals) giving them many uses in industrial chemistry. In particular, polyphosphates have long been used to prevent calcification; for example, sodium polyphosphate was first used in the Calgon® water softener in the 1930s. Pyrophosphate and related polyphosphates, such as hexametaphosphate, have also been extensively used as toothpaste additives to prevent dental calculus formation and as food additives. However, it was the pioneering work of Fleisch and colleagues in the 1960s that identified the ability of pyrophosphate to inhibit biomineralisation [4-7]. They discovered that pyrophosphate potently antagonises the ability of calcium to crystallise with phosphate to form hydroxyapatite ($Ca_{10}(PO_4)(OH_2)$) [5,7]. Pyrophosphate also binds stongly to the surface of hydroxyapatite crystals and blocks their ability to act as a nucleator for mineralisation therefore preventing further crystal growth [8].

This initial work helped to establish the concept that pyrophosphate is the body's own "water softener" which acts to prevent harmful soft tissue calcification and regulate bone mineralisation [8,9]. Subsequent studies using ^{32}P-labelled pyrophosphate in dogs enabled the kinetics of pyrophosphate production and elimination to be examined (**Figure 2)** [10]. This work suggested that the daily turnover of extracellular pyrophosphate in an adult human might be in the range 100mg/day, a very small amount compared with the many grams likely to be generated intracellularly during biosynthetic reactions. Early studies also revealed that the pyrophosphate in human bodily fluids, including urine, is endogenous and

does not come from dietary sources [3]. Indeed feeding large amounts of pyrophosphate did not increase levels any more than giving the same amount of inorganic phosphate. This is because pyrophosphate, like other phosphate compounds, seems to be completely hydrolysed within the intestinal tract by enzymes including alkaline phosphatase located on the brush borders of intestinal villous cells. In the 1960-70s it was thought that feeding phosphate might be effective in reducing kidney stone formation in patients; although this seemed counterintuitive it increased urinary pyrophosphate, by a mechanism that appeared to involve inhibition of its intra-renal hydrolysis [11]. Reduced levels of pyrophosphate are also found in some groups of stone formers [12].

Pyrophosphate is found in mineralised tissues (e.g. teeth and bone) at concentrations representing approximately 0.5% of the total phosphate content [13,14]. The intracellular concentrations have been difficult to determine, not least because of compartmentalisation, but are likely to be at least tenfold lower than that of inorganic phosphate. Interestingly in platelets, pyrophosphate is found in dense granules which are released during blood clotting [1]. This is important because serum levels of pyrophosphate produced *in vitro* can be several-fold higher than plasma concentrations, and this has previously led to misinterpretation of circulating levels of pyrophosphate in human diseases.

Deposits of pyrophosphate as calcium salts occur in humans, such as in the disease chondrocalcinosis, but also in nature. For example, deposits of amorphous calcium pyrophosphate mixed with calcium phosphates are found in the hepatopancreas of snails where they are thought to selectively accumulate metal irons, and have been used as monitors of toxic metals like cadmium, zinc and mercury in the environment [15].

Much remains to be learnt about the role of pyrophosphate in biology and mineralisation. For example, high pyrophosphate levels (>100µM) are found in milk where it may help to keep the extremely high concentrations of calcium and phosphate in a colloidal state and prevent them from precipitating out (RGG Russell, *unpublished observation*). This article is dedicated to the memory of Herbert R Fleisch and William F Neuman, whose discoveries laid the foundations for understanding the role of pyrophosphate in mineralisation. It will summarise our current understanding of how this simple molecule regulates mineralisation.

Generation and regulation of extracellular pyrophosphate

In vivo a balance between the rate of production and hydrolysis ensures the concentration of extracellular pyrophosphate is carefully regulated (**Figure 3**). Extracellular nucleotides such as ATP are thought to be an important source of the pyrophosphate present outside cells. The (NPP) ecto-nucleotide pyrophosphatase/phosphodiesterase family of enzymes catalyse the hydrolysis of ATP/UTP to the corresponding monophosphate and pyrophosphate. NPPs are widely expressed and highly conserved between species. In humans, there are 7 members of the NPP family [16] each with different expression and substrate specificity. NPP1 (or PC-1), NPP2 (autotoxin) and NPP3 (B10) have been particularly well characterised with regard to their roles in pyrophosphate generation.

The intracellular ATP concentration is between 2-5mM. Following membrane damage all cells can release ATP into the extracellular environment; however, controlled release has also been

demonstrated from numerous excitatory and non-excitatory cells (e.g. bone cells [17], endothelial and epithelial cells [18,19], vascular smooth muscle cells [20]). Following release, ATP can act in an autocrine/paracrine manner to influence local purinergic signalling but it is also rapidly broken down by ecto-nucleotidases including NPPs. To date several different processes have been implicated in mediating ATP release (e.g. connexin hemichannels, the P2X7 receptor) but the predominant mechanism appears to be vesicular exocytosis (see review [21]). The extent of cellular ATP release can also be influenced by external stimuli such as hypoxia [22,23], mechanical stress [24,25] and vitamin D [26]. Since ATP hydrolysis is a key source of extracellular pyrophosphate, factors which regulate ATP release may also indirectly affect pyrophosphate levels and thus the local rates of mineralisation. However, at present the relationship between controlled ATP release and the extracellular pyrophosphate concentration is poorly investigated and presents an interesting area for future study.

The membrane protein ANK (progressive ankylosis or ANKH), which is thought to facilitate transport of pyrophosphate from the intra-to-extracellular environment, may also contribute to extracellular pyrophosphate levels [27]. However, since the intracellular pyrophosphate concentration is only in the micromolar range the relative contribution of ANK to extracellular pyrophosphate levels is likely to be smaller than the breakdown of ATP by NPPs [28]. At present, the biological role and function of ANK remains unclear. Although mutations are found in patients with 'pyrophosphate' diseases such as chondrocalcinosis [29,30], loss of function mutations are also found in other skeletal disorders, notably craniometaphysial dysplasia (CMD) [31,32]. This autosomal dominant condition is characterised by abnormal bone mineralisation leading to craniofacial bone thickening, widened long-bone metaphyses and increased cortical thickness. At present any role of pyrophosphate in CMD remains obscure.

Alkaline phosphatases, of which there are four, are broad spectrum ecto-phosphatases that hydrolyse numerous phosphate containing molecules [16]. In particular they have pyrophosphatase activity and so will break down pyrophosphate to two phosphates. It is important to note that alkaline phosphatase, as its names implies, is usually assayed at high pH, but its kinetics are different at physiological pH where the Km for substrates like pyrophosphate is very low, and it is capable of 'completely' hydrolysing pyrophosphate [16]. Thus the addition of excess alkaline phosphatase to plasma or urine results in reduction of pyrophosphate to unmeasurable levels.

Biological mineralisation and the role of pyrophosphate as an inhibitor

As originally highlighted by Fleisch and Neuman [4-7], body fluids are supersaturated with respect to calcium and phosphate, and mineralisation is facilitated by the presence of nucleating agents. Their pioneering studies identified collagen as an important nucleator, and they showed that the maintenance of supersaturated levels of calcium and phosphate was achieved by the presence of inhibitors. The key inhibitor was destroyed by alkaline phosphatase and proved to be pyrophosphate [4-7].

The concentrations of extracellular calcium and phosphate are major determinants of mineralisation both within the skeleton (bone and cartilage) and other tissues. In clinical disorders, such as vitamin D deficiency, skeletal mineralisation is impaired by low calcium and phosphate levels [33]. Conversely

when calcium or phosphate levels are high, as in renal failure, ectopic mineralisation can occur. Plasma phosphate levels vary physiologically over a wider range than calcium and are significantly influenced by dietary intake [34]. They are also regulated by renal excretion, which in turn is modulated by several factors, including parathyroid hormone (PTH), growth hormone and FGF23 [34].

Pyrophosphate as an inhibitor of bone mineralisation

The inhibitory actions of pyrophosphate have been extensively studied in bone. It is now thought that the phosphate-to-pyrophosphate ratio within the bone microenvironment is a fundamental regulator of skeletal mineralisation (see review [35]). Osteoblasts express at least 3 members of the NPP family (NPP1, 2, 3), and of these, NPP1, is thought to be the most important in pyrophosphate generation [36-38]. Tissue non-specific alkaline phosphatase (TNAP) is the only alkaline phosphatase implicated in mineralisation and is the key enzyme involved in pyrophosphate breakdown [35,36]. Previous work has suggested that the opposing actions of NPP1 and TNAP may be critical in determining local extracellular phosphate and pyrophosphate levels [28,37]. Deletion or inactivation of one of these enzymes has a significant effect on the skeleton (see reviews [35,39]). For example, patients with hypophosphatasia lack TNAP resulting in increased pyrophosphate levels and impaired bone mineralisation [40,41]. In contrast, the human disease ossification of the posterior longitudinal ligament of the spine (OPLL), which is characterised by ectopic calcification of spinal ligaments, is caused by a mutation in NPP1 that leads to a reduced enzyme activity [42]. The treatment of these diseases remains challenging, but there has been remarkable recent success in treating hypophosphatasia with TNAP enzyme replacement therapy [43].

NPP1

The important role of NPP1 in pyrophosphate generation and skeletal mineralisation has been highlighted by three different mouse models; the naturally occurring NPP1 “knockout” termed the tip-toe walking (*ttw/ttw)* mouse, the genetically altered NPP1 knockout (*Enpp1*$^{-/-}$) and the alternative *Enpp1*^{asj} knockout. The *ttw/ttw* model displays ossification of the spinal ligaments, peripheral joint hyperstosis and calcification of articular cartilage [42]. The phenotype of *ttw/ttw* mice has similarities to OPLL. To date the *Enpp1*$^{-/-}$ model has been studied in the most detail; these animals display aberrant calcification of the spine, joints, tendons and extra-skeletal cartilage which progressively worsens with age and is associated with a reduction in movement and altered gait (**Figure 4**) [36,44-46]. Surprisingly, given the lower extracellular pyrophosphate levels, *Enpp1*$^{-/-}$ mice exhibit reduced trabecular and cortical bone in the appendicular skeleton and decreased bone strength [45-47]. The reasons for this unexpected phenotype are unclear but could involve factors such as decreased movement, increased levels of FGF-23, a regulator of phosphate metabolism, and sclerostin, an inhibitor of bone mineralisation [45,46,48] and diminished blood flow to bone owing to mineral occlusion of the blood vessel channels in bone [46]. *Enpp1*^{asj} mice, which have a different genetic background to *Enpp1*$^{-/-}$ animals, also display many of the same phenotypic characteristics such as widespread ectopic calcification [49].

ANK

Like NPP1 the postulated function of ANK is to increase extracellular pyrophosphate albeit via a different mechanism. In *ank/ank* mice, a mutation in the C-terminal cytosolic domain of ANK attenuates pyrophosphate transport to the extracellular environment [27]. These animals display abnormal pyrophosphate levels, joint calcification and destruction, impaired gait and vertebral fusion characteristic of ankylosing spondylitis [27]. Interestingly, a comparative study reported that the ectopic mineralisation in *ank/ank* mice is less severe than in *Enpp1*$^{-/-}$ animals suggesting that NPP1 is more important in extracellular pyrophosphate generation [28].

Controlling pyrophosphate levels in bone

Regulation of NPP1, TNAP and ANK (and consequently pyrophosphate levels) expression and activity is essential to prevent hypo- or hypermineralisation. Many signalling pathways are likely to be involved but one of the most interesting is the apparent ability of pyrophosphate to control its own production. Exogenous pyrophosphate down-regulates *Enpp1* and *Ank* expression in osteoblasts [28,38]. ATP and UTP also inhibit *Enpp1* expression although it is unclear whether this is due to purinergic signalling or because of an NPP1-mediated increase in pyrophosphate [38]. Nevertheless these data suggest the presence of a negative feedback pathway by which pyrophosphate regulates gene expression. How pyrophosphate activates intracellular signalling pathways is unknown. Its size and charge means that it cannot passively cross the cell membrane and this raises the intriguing possibility of a pyrophosphate receptor or sensor (**Figure 3**).

Whilst pyrophosphate can inhibit *Enpp1* expression increased phosphate levels can induce it [50]. Other factors which can regulate extracellular pyrophosphate via actions on TNAP, NPP1 and/or ANK include neurofibromin [51], acidosis [52,53], hypoxia-inducible factor proteins [54], FGF2 [55,56] and vitamin D [57].

Pyrophosphate and osteocytes

Osteocytes, the most abundant cell type in bone [58], reside within lacunae surrounded by mineralized matrix. These cells release numerous soluble factors which regulate osteoblast and osteoclast function thereby allowing them to control bone remodeling [59]. Since osteocytes are embedded within bone they must be capable of preventing over-mineralisation of their lacunae (which could potentially compromise cell viability and function). ATP, which is released by all bone cells including osteocytes [22,25,60-65], is an important source of pyrophosphate in bone [17,66]. Previous work has shown that endogenous ATP released by osteoblasts acts as an important local brake on mineralisation, an effect mediated by both purinergic signalling and the breakdown to produce pyrophosphate [38,67,68]. Detailed analysis of cortical bone revealed that *Enpp1*$^{-/-}$ mice display a significant reduction in the size and number of osteocyte lacunae, an effect which was attributed to reduced pyrophosphate levels [46]. Hajjawi *et al* [46] suggested that under normal conditions the ATP constitutively released by osteocytes is broken down by NPP1 to pyrophosphate which then acts to maintain lacunar size [46]. Regulation of lacunar size during lactation, when demand for calcium release is high, may involve similar mechanisms [69]. Further work is required to fully understand the role of pyrophosphate in osteocytes.

Pyrophosphate as a regulator of soft tissue calcification

Since soft tissue calcification usually results in severe pathological changes robust regulatory mechanisms are in place to prevent it. NPP1 appears to be particularly important in generating the extracellular pyrophosphate needed to prevent unwanted soft tissue calcification, as illustrated by *Enpp1*$^{-/-}$ mice which display widespread and dramatic calcification of tissues including the aorta, kidney, ear pinna, trachea, whisker follicles, cartilage and tendons [45,46,48] (**Figure 4**).

Pyrophosphate and cartilage mineralisation

Normal joints contain both articular cartilage, which must remain unmineralised in order to function correctly, and calcified cartilage which forms the interface between articular cartilage and the underlying subchondral bone. To maintain joint health and integrity, cartilage calcification needs to be tightly controlled and restricted to specific regions. Chondrocytes, the resident cell type in cartilage, release ATP constitutively [70], display high levels of NPP1 activity and can produce large amounts of extracellular pyrophosphate [71,72]. In the degenerative joint disease osteoarthritis (OA), aberrant articular cartilage calcification may occur and damage the surrounding tissue [73]. NPP1 levels are reported to be lower in cartilage from patients with severe OA [74] and *Enpp1* polymorphisms have been associated with hand OA [75]. Furthermore, calcium deposits and OA-like changes have been described in the articular cartilage of *ttw/ttw* mice [74,76]. Thus, it appears that NPP1 and pyrophosphate play an important but not yet fully defined role in preventing pathological cartilage calcification.

Although pyrophosphate may act to protect cartilage against inappropriate mineralisation, in excess it may be detrimental because it can promote the formation of calcium pyrophosphate dihydrate (CPPD) crystals and the development of chondrocalcinosis. This condition occurs in familial forms but is also extremely common in ageing populations, where it can lead to significant morbidity [77]. It has been suggested that elevated pyrophosphate levels may involve ANK since protein expression is higher in patients with CPPD deposits and activating mutations in the *Ankh* gene have been associated with inherited forms of chondrocalcinosis [78,79].

Pyrophosphate and vascular calcification

Vascular calcification refers to the pathological deposition of calcium phosphate mineral, most often hydroxyapatite, in arteries, heart valves and cardiac muscle. It shares some outward similarities with bone mineralisation and is associated with a phenotypic transdifferentiation of vascular smooth muscle cells (VSMCs) towards a more osteoblast-like phenotype [80].

Vascular calcification is particularly common in patients with advanced chronic kidney disease, where it is inversely correlated with circulating pyrophosphate levels [81,82]. Early work reported that aortic calcification was inhibited by pyrophosphate injections [83]. This idea is supported by a recent investigation which found daily pyrophosphate injections reduced the incidence and amount of uraemia-induced vascular calcification without adversely effecting bone [84]. Taken together these studies suggest a potential therapeutic use of pyrophosphate.

Mutations in the *Enpp1* gene are associated with a rare autosomal recessive condition called generalised arterial calcification of infancy (GACI) [85,86]. Sufferers of this condition usually die in

infancy because of substantial vascular calcification. Consistent with an inhibitory role, *Enpp1*$^{-/-}$ mice exhibit significant vascular calcification *in vivo* and *Enpp1*$^{-/-}$ VSMCs have a reduced ability to generate pyrophosphate from ATP leading to increased calcification *in vitro* [45,87]. *Enpp1*$^{-/-}$ VSMCs also display higher expression of chondrogenic, osteoblastic and osteocytic markers [88]. Furthermore, a recently published study has shown that subcutaneous administration of an NPP1 fusion protein prevents vascular calcification in *Enpp1*^{asj} mice [89]. *In vitro* studies have additionally shown that, by hydrolysing released ATP, NPP1 is a key source of pyrophosphate in VSMC cultures [20,90]. ANK may also contribute to extracellular pyrophosphate levels needed to prevent vascular calcification although evidence suggests that it plays a less important role than NPP1 [20,87].

Pyrophosphate and pseudoxanthoma elasticum

Pseudoxanthoma elasticum is an autosomal recessive condition characterised by reduced plasma pyrophosphate levels and progressive ectopic mineralisation of the skin, eyes and arteries [91-93]. It is primarily caused by inactivating mutations in the ATP-binding cassette subfamily C member 6 (*ABCC6*) gene [94]. ABCC6 is primarily expressed in the liver and mediates ATP release from hepatocytes. *Abcc6*$^{-/-}$ knockout animals exhibit the symptoms of pseudoxanthoma elasticum and display a 40% reduction in plasma pyrophosphate levels [91]. Studies using these animals have suggested that ATP release is impaired in cells lacking ABCC6 and that the lack of substrate for NPP1 results in lower circulating pyrophosphate levels and the development of pseudoxanthoma elasticum [66]. Interestingly, it has recently been identified that polymorphisms in the TNAP, NPP1 and ANK genes are risk factors for developing pseudoxanthoma elasticum [95].

Pyrophosphate and Hutchinson-Gilford progeria syndrome

Hutchinson-Gilford progeria syndrome is a rare disorder characterised by high levels of atherosclerosis and vascular calcification [96,97]. Patients with this condition express a mutant form of lamin A, called progerin. Knock-in mice overexpressing progerin have reduced circulating pyrophosphate levels and vascular calcification [98]. The reduction in pyrophosphate levels in these animals was attributed to increased alkaline phosphatase activity and decreased extracellular ATP levels [98].

Pyrophosphate: the mechanism of action

Following considerable work in the 1960-1970s, the direct effects of pyrophosphate on hydroxyapatite formation are now well established. It inhibits de novo crystal formation, and retards the conversion of amorphous calcium phosphates to crystalline apatites. However, the physicochemical interactions between pyrophosphate and calcium phosphates are complex. For example, in the original studies of Fleisch and Neuman low pyrophosphate and polyphosphate concentrations promoted mineralisation in cultured chick embryo femurs whilst higher concentrations had the expected inhibitory effect [99].

Accumulating evidence suggests that pyrophosphate may also exert non-physicochemical effects including regulation of its own production [28,38] . Osteopontin is a secreted glycoprotein which limits hydroxyapatite formation and deposition [100,101]. Pyrophosphate can induce osteopontin in osteoblasts via the MAPK signalling pathway [28,102] and both *Enpp1*$^{-/-}$ and *ank/ank* mice display reduced osteopontin expression in osteoblasts and decreased serum levels of the protein [28,44,45].

Thus, pyrophosphate-induced osteopontin could represent an important mechanism to prevent ectopic calcification.

In addition to the known inhibitory effects of phosphate on TNAP, direct inhibitory actions of pyrophosphate on TNAP have also been suggested. Earlier work indicated that, in the presence of a second substrate such as β-glycerophosphate, pyrophosphate can cause a conformational change in TNAP which inhibits the enzyme thereby reducing pyrophosphate hydrolysis [102] .

Circulating pyrophosphate

The plasma concentration of pyrophosphate is reported to be in the range 1-6μM/litre [103]. However, the tissue source of circulating pyrophosphate remains the subject of some debate. Some evidence indicates that the skeletal system maybe a significant source [36], however, more recent work suggests that other tissues such as the liver could also contribute [66]. Nevertheless, it is becoming apparent that systemic pyrophosphate levels play a key role in preventing unwanted soft tissue calcification. As already mentioned plasma pyrophosphate is reduced in patients with vascular calcification [81,82]. Furthermore, recent work demonstrated that transplanting *Enpp1*$^{-/-}$ aortas into wildtype littermates stopped vascular calcification from developing; conversely, if wildtype aortas were transplanted into *Enpp1*$^{-/-}$ animals they began to calcify [48]. Thus it has been suggested that systemic levels of pyrophosphate could represent a measurable risk factor for vascular calcification [48].

Pyrophosphate and bisphosphonates

Bisphosphonates (BPs), which are potent inhibitors of osteoclast activity, are widely used to prevent the bone loss associated with conditions such as osteoporosis, Paget's disease and metastatic bone disease (see reviews [104,105]). They are chemically stable analogues of pyrophosphate, in which the central oxygen atom is replaced by carbon to form a P-C-P moiety; variations in the R1 and R2 side chains off the central carbon produce the individual bisphosphonates [104,106]. Like pyrophosphate, BPs bind strongly to bone mineral and inhibit the formation and propagation of hydroxyapatite crystals [107]. The binding affinity of the different BPs for hydroxyapatite, and hence their uptake and persistence, is influenced by their R1 and R2 groups [104,108] (**Figure 1**). Despite the similarities, there are some critical differences between pyrophosphate and BPs. Firstly, pyrophosphate has to be injected as it is ineffective orally because of hydrolytic destruction within the gut; BPs are effective by mouth despite being poorly absorbed. Secondly, pyrophosphate does not inhibit bone resorption, whereas this is the key pharmacological action of BPs when used to treat clinical disorders characterised by excessive resorption [100]. BPs are very effective drugs with more than 40 years of clinical use, and have proven to be remarkably safe, with an excellent benefit to risk ratio [109]. Despite this there is a considerable literature on the possible adverse effects, namely osteonecrosis of the jaw (ONJ) and atypical femoral fractures. Such events are very rare and their pathogenesis remains unclear. Claims that the similarities between pyrophosphate and BPs might explain these phenomena [110] are speculative and without scientific foundation, since pyrophosphate and polyphosphates do not inhibit bone resorption [106,111].

It has long been known that BPs can inhibit mineralisation in both bone and cartilage, as well as in soft tissues. This proved to be an issue with the early BPs such as etidronate, but with the BPs currently used the therapeutic window between inhibition of mineralisation and bone resorption differs by several orders of magnitude, so this is no longer a clinical problem. There are more recent studies suggesting that BPs may inhibit bone formation and mineralisation, an effect which may, because of the structural similarities with pyrophosphate, involve direct physicochemical effects on hydroxyapatite crystal propagation [112,113]. These inhibitory actions on mineralisation may prove beneficial if BPs are ever to be used as potential therapeutics for treating conditions associated with unwanted calcification such as vascular calcification and GACI [114-117].

BPs have many clinical uses in bone diseases and many non-skeletal effects based on their ability to inhibit protein prenylation. In a mouse model of Hutchinson-Gilford progeria syndrome, a combination of a statin with zoledronate was able to markedly extend lifespan and offset many of the tissue ageing effects [118]. These observations have led to the use of these drug combinations in patients with Hutchinson-Gilford progeria syndrome, with apparently promising results [119].

Concluding remarks

Our understanding of how pyrophosphate prevents unwanted mineralisation has advanced considerably from the early seminal work describing its physicochemical effects on hydroxyapatite formation. It is now clear that numerous proteins are involved in the formation, transport and hydrolysis of pyrophosphate and defects in any of these can have profound effects on the level of mineralisation. Hydrolysis of ATP appears to be the key source of pyrophosphate and further studies to determine if alterations in controlled ATP release indirectly influence the extracellular pyrophosphate concentration are warranted. Additional work is also required to establish the mechanisms by which pyrophosphate can induce intracellular signalling pathways and whether it can be used therapeutically.

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Figure legends

Figure 1. ***The chemical structure of phosphate, pyrophosphate, polyphosphates and bisphosphonates***

Figure 2. ***Systemic extracellular metabolism of pyrophosphate***

Studies using ^{32}P-pyrophosphate injected into dogs showed how extracellular pyrophosphate is produced and eliminated systemically. Figure is adapted from Jung *et al* [10].

Figure 3. ***Regulation of extracellular pyrophosphate levels and mineralisation***

The intracellular ATP concentration is 2-5mM. ATP is released from most cells via controlled mechanisms such as vesicular exocytosis. Once outside the cell, ATP is rapidly broken down by NPP1 to produce AMP and pyrophosphate. The membrane protein ANK may directly transport pyrophosphate, where it is found at micromolar levels, from inside to outside the cell contributing to extracellular pyrophosphate levels. Extracellular pyrophosphate acts to prevent mineralisation by preventing hydroxyapatite formation and growth. It can also regulate gene expression suggesting the presence of a yet unknown pyrophosphate receptor/sensor. TNAP hydrolyses pyrophosphate to two phosphate molecules which may contribute to mineralisation in association with the much higher concentrations of phosphate available in extracellular fluids.

Figure 4. ***Enpp1$^{-/-}$ mice display widespread ectopic calcification***

MicroCT images (9µm resolution) showing the ectopic calcification in 20 week-old *Enpp1$^{-/-}$* mice (highlighted by the arrows). Images obtained using a SkyScan 1176 high resolution *in vivo* scanner (Bruker MicroCT, Kontich, Belgium).

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Figure 1

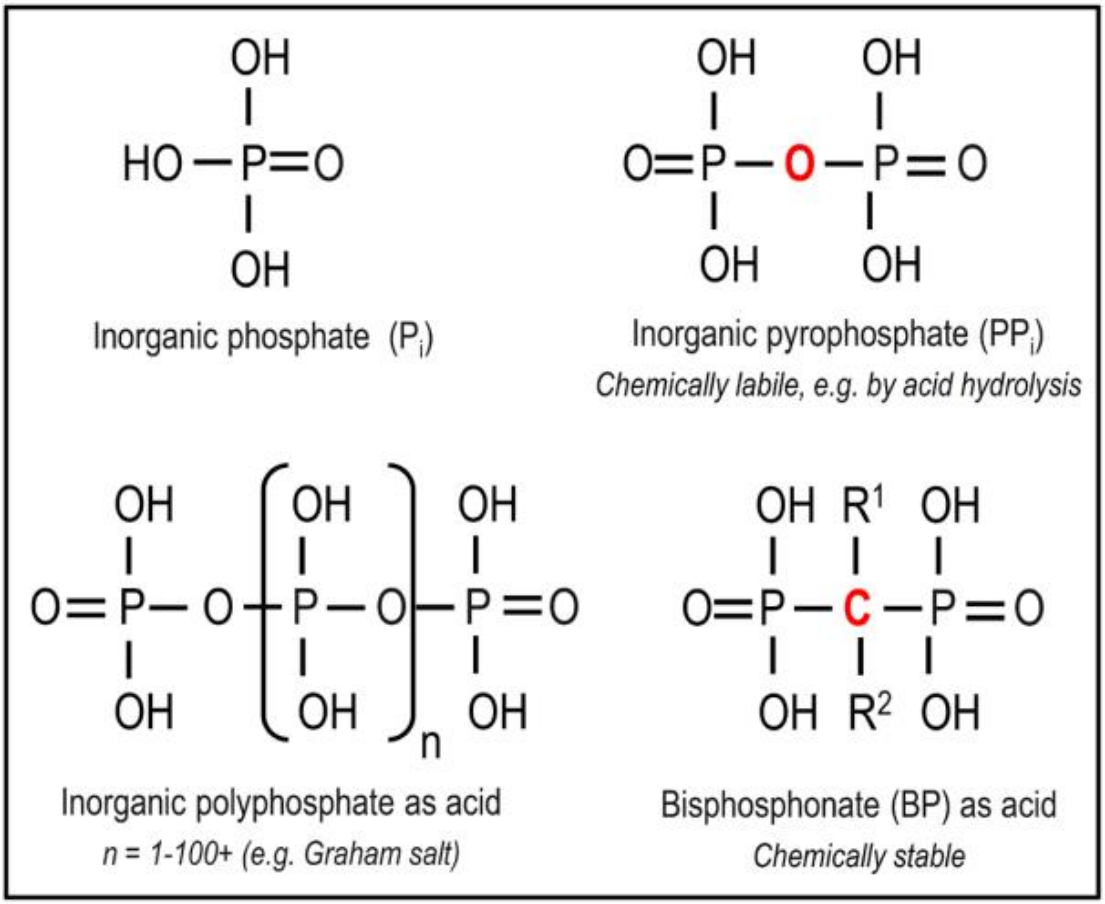
Inorganic phosphate (P_i)
Inorganic pyrophosphate (PP_i)
Chemically labile, e.g. by acid hydrolysis
Inorganic polyphosphate as acid
n = 1-100+ (e.g. Graham salt)
Bisphosphonate (BP) as acid
Chemically stable

Figure 2

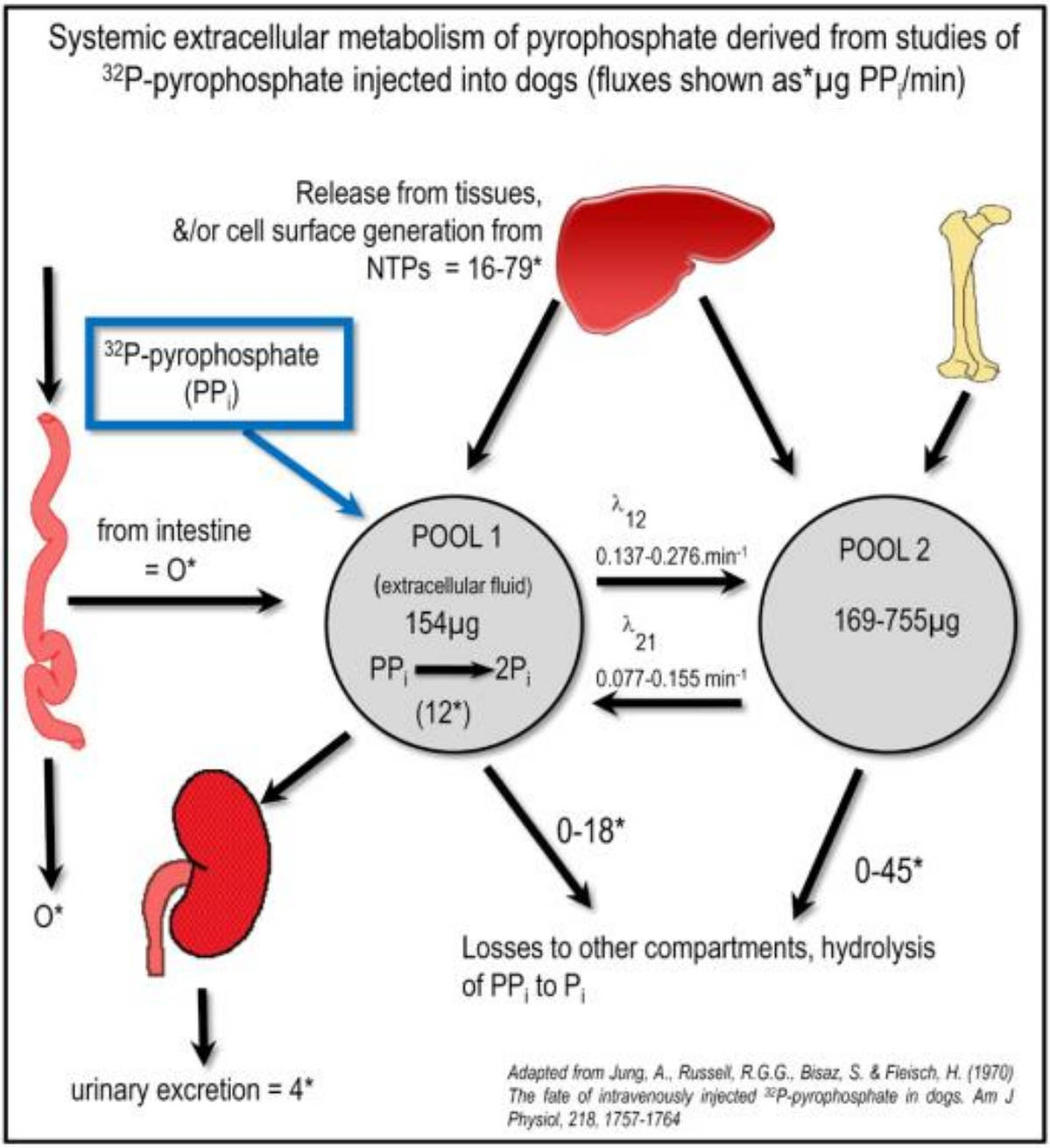
Systemic extracellular metabolism of pyrophosphate derived from studies of ^{32}P-pyrophosphate injected into dogs (fluxes shown as*µg PP$_i$/min)
Release from tissues, &/or cell surface generation from NTPs = 16-79*
^{32}P-pyrophosphate (PP$_i$)
from intestine = O*
POOL 1
(extracellular fluid)
154µg
PP$_i$ → 2P$_i$
(12*)
λ_{12}
0.137-0.276.min^{-1}
λ_{21}
0.077-0.155 min^{-1}
POOL 2
169-755µg
O*
0-18*
0-45*
Losses to other compartments, hydrolysis of PP$_i$ to P$_i$
urinary excretion = 4*
Adapted from Jung, A., Russell, R.G.G., Bisaz, S. & Fleisch, H. (1970) The fate of intravenously injected ^{32}P-pyrophosphate in dogs. Am J Physiol, 218, 1757-1764

Figure 3

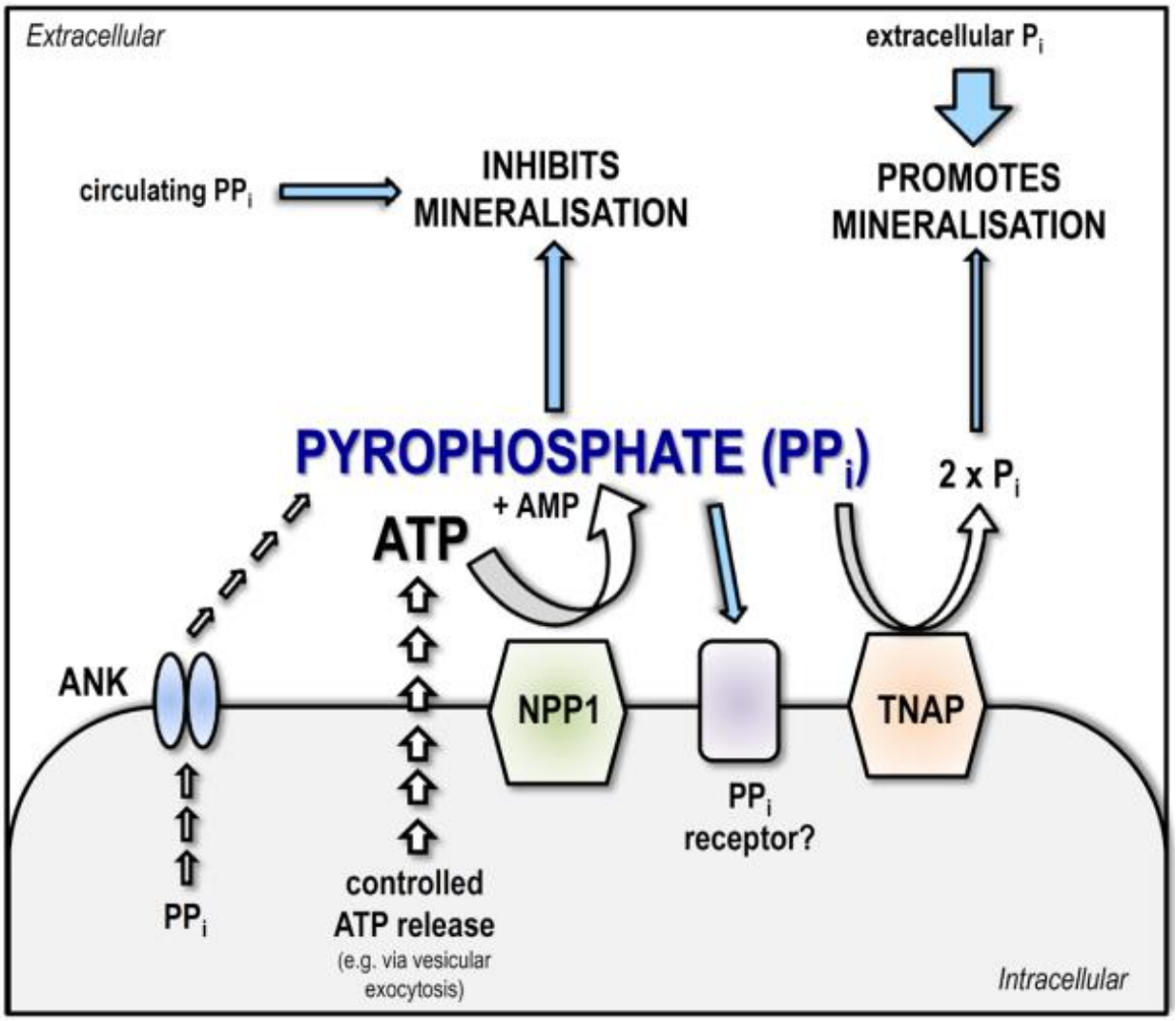
Extracellular
extracellular P_i
circulating PP_i
INHIBITS MINERALISATION
PROMOTES MINERALISATION
PYROPHOSPHATE (PP_i)
2 x P_i
+ AMP
ATP
ANK
NPP1
TNAP
PP_i receptor?
PP_i
controlled ATP release
(e.g. via vesicular exocytosis)
Intracellular

Figure 4