===== REVIEW =====

Polyphosphate in Bone

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Abstract—Human bone-forming osteoblasts are an excellent model to investigate the multiple functions of inorganic polyphosphates (polyP) for the following reasons: 1) they contain relatively high amounts of polyP and polyP-dependent enzymes; 2) they allow the study of both general and specific functions of these polymers, and 3) medically relevant results can be expected from these studies.

Key words: polyphosphate, pyrophosphate, bisphosphonates, exopolyphosphatase, alkaline phosphatase, pyrophosphatase, mineralization, bone, osteoblasts, SaOS-2 cells

Inorganic linear polyphosphates (polyP) are polymers of orthophosphate (P_i) residues linked by energy-rich phosphoanhydride bonds. They may range in size up to several hundred P_i residues [1]. In the past these polymers have been studied mainly in bacteria and yeast (for reviews, see [2, 3]), but they are also present in higher plants and animals [4-6]. More recently, polyP was found also in human cells and tissues [7-10].

The concentrations measured for "soluble" long-chain polyP (mainly polymers of 10-50 P_i residues) and "insoluble" long-chain polyP (mainly polymers of >50 P_i residues) in different cells and extracellular fluids from human are summarized in Table 1. Extraction of "soluble" long-chain polyP and "insoluble" long-chain polyP was performed as described by Clark et al. [11, 12]. In general, the concentration of soluble long-chain polyP is higher than that of insoluble long-chain polyP. PolyP concentrations in the same range as those shown in Table 1 have been detected in different mammalian cell lines (total polyP, 25 to 120 µM; expressed in P_i residues) [8]. PolyP is also present extracellularly in human blood plasma (Table 1) and serum [9]. However, the concentration of insoluble long-chain polyP in the cell-free blood fractions is much lower than that in human peripheral blood

mononuclear cells (PBMC) and erythrocytes. It is not yet known whether the plasma polyP is synthesized within this body fluid or is due to the lysis of erythrocytes, as suggested by its smaller size. The highest amounts of polyP in humans were found in bone-forming osteoblasts [10] (Table 1).

PolyP is found in nearly all cellular compartments. It is enriched in the nucleus [8, 13, 14], mitochondria [1], lysosomes [7], and plasma membrane [15]. The nuclear polyP mainly consists of long polyP chains [14].

In many mammalian cell lines, often two or more populations of polyP with a narrow range of chain lengths are found, e.g., two polyP classes with ~150 P_i residues and 25 to 45 P_i residues exist in human leukemic HL-60 cells [14], and with 500 to 800 P_i residues and 5 to 15 P_i residues in NIH3T3, PC12, and Jurkat cells [8]. PolyP shows a high turnover rate [8]. Metabolic labeling studies revealed that $^{32}P_i$ taken up by HL-60 cells appears first in the low-molecular-weight polyP fraction (25 to 45 residues) before it is incorporated into high-molecular-weight polyP (~150 residues) [14].

Many functions have been proposed for polyP (for a review, see [16]). These functions may be different in different compartments of the cell. PolyP is assumed to act as: 1) storage molecule for energy-rich phosphate; 2) chelator for Ca^{2+} or other divalent cations; 3) counter-ion for basic amino acids; 4) regulator of intracellular level of adenylate nucleotides; 5) component of complexes with poly-β-hydroxybutyrate and Ca^{2+} in cell membranes; 6) donor of P_i for certain sugar kinases, and 7) modulator of cellular stress response.

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Table 1. Concentration of long-chain polyP in human cells and blood plasma*

Cells or blood fraction	PolyP class	PolyP content**	
		P_i, µM	pmoles/mg protein
Osteoblasts***	soluble long-chain	394.3 ± 30.6	4331 ± 336
	insoluble long-chain	133.8 ± 15.2	1469 ± 167
Gingival cells	soluble long-chain	141.3 ± 15.3	1605 ± 174
	insoluble long-chain	14.9 ± 5.1	170 ± 58
PBMC	soluble long-chain	56.0 ± 2.2	622 ± 24
	insoluble long-chain	28.9 ± 7.0	321 ± 78
Erythrocytes	soluble long-chain	71.2 ± 14.7	918 ± 190
	insoluble long-chain	28.1 ± 4.1	362 ± 53
Plasma	soluble long-chain	48.7 ± 7.0	641 ± 92
	insoluble long-chain	2.5 ± 1.9	33 ± 25

  * Extraction of "soluble" long-chain polyP and "insoluble" long-chain polyP was performed as described by Clark et al. [11]. Concentrations of "soluble" (10-50 P_i residues) and "insoluble" long-chain polyP (>50 P_i residues) are presented (data from [10]).
 ** Values are the means (± SD) from three independent determinations. Concentrations are based on the volume of the pelleted cells.
*** Unstimulated osteoblasts.

OSTEOBLAST MODEL

Osteoblasts are bone-forming cells which are derived from pluripotent mesenchymal stem cells. They synthesize and secrete most of the molecules of the bone matrix, e.g., type I collagen and proteoglycans. They possess a high content of alkaline phosphatase (a marker enzyme for osteoblasts), which is involved in mineralization.

Osteoblast differentiation can be subdivided into the following phases that are associated with the sequential expression of genes involved in the biosynthesis, organization, and mineralization of the bone extracellular matrix (reviewed in [17], Fig. 1): proliferation phase, matrix maturation phase, mineralization phase, and apoptosis phase.

Proliferation phase. In this phase, genes required for proliferation (e.g., c-*fos*) and the progress of the cell cycle (e.g., cyclins, histones) as well as the genes associated with the regulation of the biosynthesis of the extracellular matrix (e.g., type I collagen, transforming growth factor β) or encoding cell adhesion proteins (e.g., fibronectin) are expressed.

Matrix maturation phase. In this phase the expression of genes involved in maturation and organization of the bone extracellular matrix (e.g., alkaline phosphatase) is upregulated, allowing mineralization.

Mineralization phase. In this phase, the expression of genes associated with the ordered deposition of hydroxyapatite (osteopontin and osteocalcin) occurs.

Apoptosis phase. An increase in type I collagen and collagenase gene expression, apoptotic cell death, and compensatory proliferative activity are observed.

The osteoblast model is a suitable cell system to study the most likely multiple functions of polyP; using this model it is possible to investigate: differential gene expression of polyP metabolic enzymes; effect of modulators on these enzymes like vitamin D, corticosteroids, or cytokines; possible function of polyP in signal transduction and membrane transport; extracellular function(s) of polyP (involvement in the mineralization process); and pathophysiology of polyP metabolism (pathogenesis of bone diseases).

MODULATION OF PolyP METABOLISM IN OSTEOBLASTS

It is known that osteoblasts respond to 1α,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$), glucocorticoids, and growth factors. They possess high levels of alkaline phosphatase, which participates in mineralization. 1,25(OH)$_2$D$_3$ is a stimulator of bone resorption; in mature osteoblasts it increases the expression of genes that are associated with the mineralization process, e.g., osteocalcin, resulting in an enhanced mineralization. On the other hand, expression of osteocalcin is suppressed by dexamethasone. However, combined treatment of osteoblasts with 1,25(OH)$_2$D$_3$ and dexamethasone has been shown to result in a synergistic increase in transcriptional activity [17].

We found that polyP metabolism in human osteoblasts is modulated by stimulators of osteoblast proliferation and differentiation [10]. Combined treatment of the cells with dexamethasone, β-glycerophos-
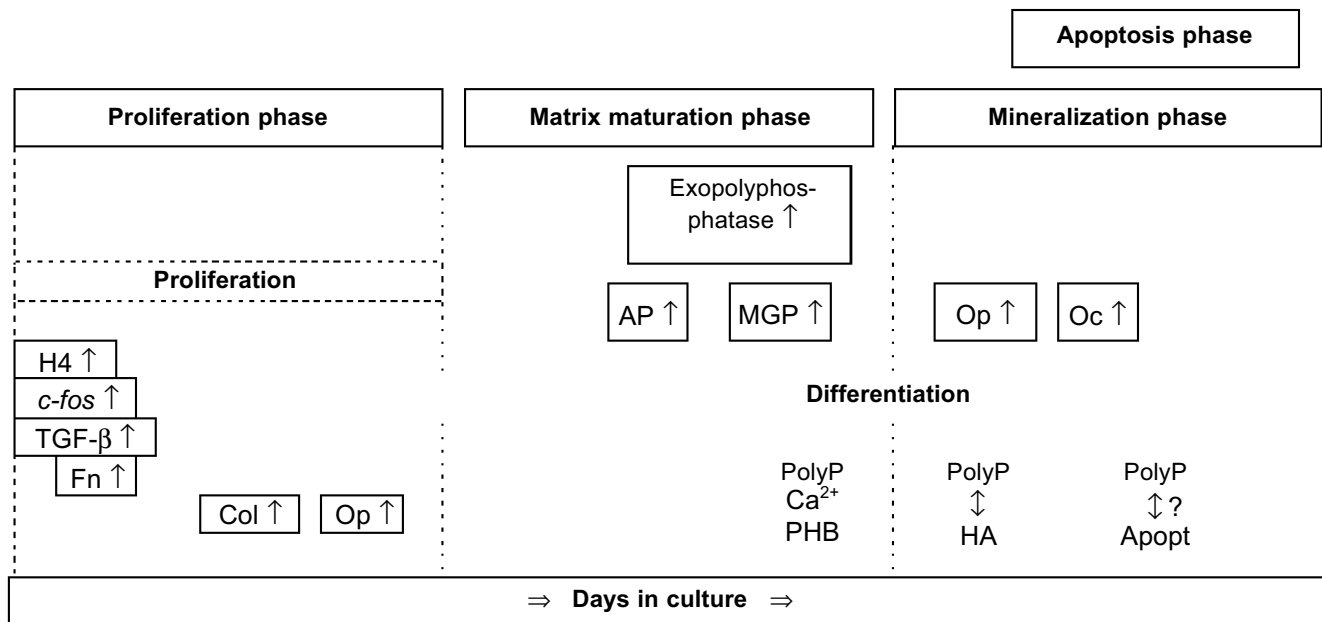
Fig. 1. Osteoblast development. Time-dependent expression of growth- and differentiation-dependent proteins. H4, histone H4; Col, collagen; Fn, fibronectin; Op, osteopontin; AP, alkaline phosphatase; MGP, matrix Gla protein; Oc, osteocalcin; PHB, polyhydroxybutyrate; HA, hydroxyapatite; Apopt, apoptosis.

phate, epidermal growth factor (EGF), and ascorbic acid results in a strong decrease in polyP content. This decrease in polyP content is mainly caused by a decrease in the amount of soluble long-chain polyP (Fig. 2). The
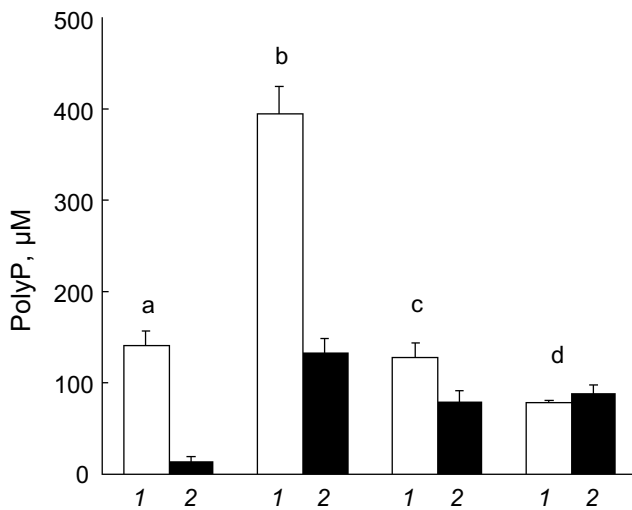

Fig. 2. Changes in polyP content of human osteoblasts after treatment with stimulators of osteoblast proliferation and differentiation. The amount of soluble long-chain polyP (*1*) and insoluble long-chain polyP (*2*) was determined in unstimulated osteoblasts (b) and in osteoblasts subjected to combined treatment with dexamethasone, β-glycerophosphate, EGF, and ascorbic acid for 6 days (c), or to combined treatment with the same additives for 3 days in the absence and then for 3 days in the presence of $1,25(OH)_2D_3$ (d). For comparison, the polyP content of gingival fibroblasts (a) is shown. Results are means ± SD of three independent experiments.

amount of this polyP fraction but not the amount of insoluble long-chain polyP, further decreases after additional treatment of the cells with $1,25(OH)_2D_3$ (Fig. 2). The decrease in polyP content during treatment with dexamethasone, β-glycerophosphate, EGF, and ascorbic acid is accompanied by a decrease in exopolyphosphatase activity [10]. However, additional treatment with $1,25(OH)_2D_3$ results in a significant increase in the enzyme activity [10].

Similar effects of dexamethasone and $1,25(OH)_2D_3$ were observed for osteoblast inorganic pyrophosphatase [10], alkaline phosphatase [10], and ectonucleoside triphosphate pyrophosphatase activity [18, 19]. The activities of inorganic pyrophosphatase and alkaline phosphatase are markedly reduced after combined treatment of the cells with dexamethasone, β-glycerophosphate, EGF, and ascorbic acid, paralleling the decrease in exopolyphosphatase activity. Again, additional treatment with $1,25(OH)_2D_3$ causes an increase in enzyme activity [10].

The ectonucleoside triphosphate pyrophosphatase, which is like alkaline phosphatase located on the cell surface, is assumed to serve in the generation of extracellular PP_i in bone [20]. The activity of this enzyme is modulated by dexamethasone and $1,25(OH)_2D_3$. Dexamethasone causes a decrease in enzyme activity, while $1,25(OH)_2D_3$ and transforming growth factor-β (TGF-β) induce an increase in activity [19]. On the other hand, TGF-β1 and TGF-β2 have been shown to decrease alkaline phosphatase activity. The increase in ectonucleoside triphosphate pyrophosphatase activity

and decrease in alkaline phosphatase activity induced by TGF-β may cause an increase in extracellular PP_i and a decrease in extracellular P_i concentration, resulting in a lower mineralization in bone tissue.

POTENTIAL ROLE OF PolyP IN MINERALIZATION

In bone, extracellular PP_i has been identified as a calcification inhibitor. PP_i has been shown to inhibit both the formation [21, 22] and dissolution of calcium phosphate crystals [23], and might therefore act as physiological regulator of calcification and decalcification [23]. It is assumed that such inhibitors must be removed from the site of mineralization before calcification can occur [22]. PP_i is hydrolyzed by alkaline phosphatase and inorganic pyrophosphatase activities. These enzymes can locally degrade PP_i in bone, thus allowing formation of hydroxyapatite crystals. The increase in both enzyme activities induced by $1,25(OH)_2D_3$ would be expected to result in an increased production of P_i and a decrease in PP_i concentration, thus modulating mineralization during bone formation. Indeed, bone mineralization has been found to be associated with changes in pyrophosphatase activity [24].

Long polyP chains inhibit hydroxyapatite precipitation more strongly than PP_i [22]. The efficiency of $polyP_{11}$ and $polyP_{27}$ to suppress calcium phosphate precipitation is ~10-fold higher than that of PP_i and is ~10^3-fold higher than that of ATP [22]. Our studies showed that polyP is present in bone tissue [10]. Therefore, it is reasonable to assume that polyP may act as a further regulator of calcification and decalcification in bone tissue, in addition to PP_i.

Recently, we demonstrated that treatment of cultures of human osteosarcoma cell line SaOS-2 with polyP causes a strong suppression of mineralization induced by β-glycerophosphate (Kurz et al., to be published). As shown in Fig. 3, cells grown in the presence of ascorbic acid and in the absence of β-glycerophosphate showed a small but progressive increase in calcium deposition as measured by Alizarin Red S staining. Addition of β-glycerophosphate caused a strong enhancement of the mineralization process. This increase was abolished in the presence of polyP, indicating that this polymer may act as a mineralization inhibitor.

PolyP-DEPENDENT ENZYMES IN OSTEOBLASTS

The presence of polyphosphatases, which locally destroy polyP (an inhibitor of calcium phosphate precipitation), might be necessary for bone tissue to mineralize, in addition to PP_i-degrading pyrophosphatase and
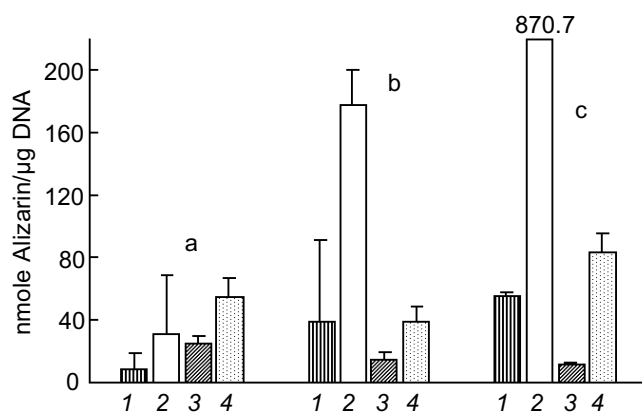
Fig. 3. Effect of polyP on mineralization of SaOS-2 cells. Human osteosarcoma SaOS-2 cells were grown to confluency (day 0) and then incubated for 4 (a), 8 (b), and 10 days (c) in the presence of 0.25 mM ascorbic acid (*1*), 0.25 mM ascorbic acid and 10 mM β-glycerophosphate (*2*), 0.25 mM ascorbic acid and $polyP_{35}$ (1 mM, expressed as P_i) (*3*), and 0.25 mM ascorbic acid, 10 mM β-glycerophosphate, and polyP (1 mM P_i) (*4*). Calcium deposition was quantified by Alizarin Red S staining. Means ± SD ($n = 3$).

alkaline phosphatase activities [10]. In addition, polyphosphatases may be involved in generation of P_i required for formation of hydroxyapatite crystals.

Exopolyphosphatase. PolyP degradation is known to be catalyzed by several endo- and exopolyphosphatases, which differ in substrate specificity and product [5, 25-30]. Exopolyphosphatase activity is also present in human osteoblasts [10]. The specific activity of the enzyme in unstimulated osteoblasts is much higher than that in other mammalian cells and tissues tested (Table 2). Exopolyphosphatase activity has also been found extracellularly, e.g., in synovial fluid (see Fig. 4) as well as in human blood plasma and serum [9].

The degradation of long-chain polyP by extracts from SaOS-2 cells and human synovial fluid is shown in Fig. 4. Both the membrane fraction (Fig. 4, lanes *2-4*) and cytosolic fraction (Fig. 4, lanes *5-7*) from SaOS-2 cells contain exopolyphosphatase activity cleaving long-chain $polyP_{750}$ under formation of P_i. In addition, a further high-molecular-weight polyP band became visible during incubation, possibly due to an endopolyphosphatase activity (see below). Exo- and endopolyphosphatase activity is also present extracellularly in synovial fluid (Fig. 4, lanes *8* and *9*).

Moreover, we found that in non-proliferating (confluent) SaOS-2 cells higher levels of exopolyphosphatase activity were present than in proliferating cells. More than 50% of the exopolyphosphatase activity in SaOS-2 cells is membrane-bound (Kurz et al., to be published).

Endopolyphosphatase. Endopolyphosphatase activities cleaving long-chain polyP have been identified in a variety of eukaryotic organisms including mammals

Table 2. Exopolyphosphatase activities in different cells, tissues, and extracellular fluids from mammals*

Cells and tissues	Polyphosphatase activity** (P_i liberated in nmole/h per mg of protein)
Rat	
liver***	48
brain***	54
Human	
osteoblasts	210
HL-60 cells	25
PBMC	15
plasma	5.5
serum	5.4

* Data are from [9, 10, 14].
** Mean values from three independent experiments; $polyP_{35}$ was used as substrate at 37°C.
*** Adult animals.

[30]. The enzyme catalyzes the degradation of long-chain polyP with formation of shorter chains up to a chain length of about 60 residues; tripolyphosphate is produced in a ratio of $polyP_{\geqslant 60}$ to $polyP_3$ of about 3 (in terms of polymer concentration) [30]. Preliminary results indicate that this enzyme is also present in bone-forming osteoblasts (see Fig. 4).

Alkaline phosphatase. Alkaline phosphatase is a well-known enzyme which is widely used in clinical diagnosis of various diseases including bone disorders. This enzyme cleaves phosphate groups from monophosphate ester substrates; in addition, it exhibits phosphotransferase and protein phosphatase activity [32]. In human, four isoforms of alkaline phosphatase exist: intestinal, placental, placenta-like (germ cell type) and tissue-nonspecific [33]. The tissue-nonspecific isoform of alkaline phosphatase is present in bone, liver and kidney; it differs from the other forms by posttranslational modification [34, 35].

The physiological function of alkaline phosphatase is still uncertain. It is assumed that the enzyme is involved in bone mineralization [36], dissolution of calcium pyrophosphate crystals [37, 38], phosphate and calcium transport [39, 40], and intestinal phosphate absorption [41]. The natural substrates of the enzyme include PP_i, β-glycerophosphate, phosphoethanolamine, and pyridoxal-5′-phosphate.

We could demonstrate that the intestinal isoform of alkaline phosphatase is able to degrade polyP molecules with a wide range of chain lengths, in addition to PP_i

[42]. As shown in Fig. 5, alkaline phosphatase from calf intestine cleaves $polyP_{750}$ with formation of P_i, apparently by a processive mechanisms. No formation of intermediate chain lengths was found in the course of the reaction. The pH optimum of the enzyme for polyP hydrolysis is in the alkaline range like that for the synthetic substrate, *p*-nitrophenylphosphate. The enzyme is also active in the absence of divalent cations, in contrast to other exopolyphophatases [16, 42], except for exopolyphosphatase I from *Tethya lyncurium* [5].

The placenta-type and tissue nonspecific isoenzymes of mammalian alkaline phosphatase were found to be unable to degrade polyP under the tested conditions [42]. Therefore, exopolyphosphatase activity in osteoblasts [10, 43] cannot be due to tissue nonspecific alkaline phosphatase activity present in these cells. However, it cannot be excluded that the assay conditions used were not appropriate to allow polyP hydrolysis by this isoform.
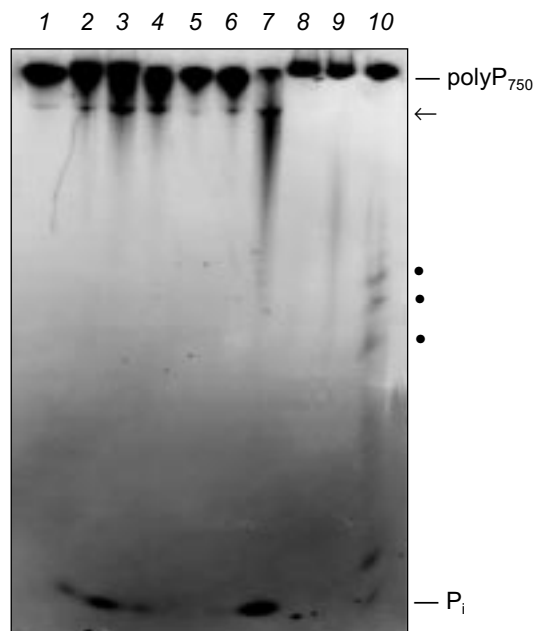

Fig. 4. Degradation of long-chain polyP by cell extracts from human SaOS-2 osteosarcoma cell line and human synovial fluid. The substrate, $[^{32}P]polyP_{750}$ (*1*), was synthesized by recombinant *E. coli* polyphosphate kinase (a kind gift from Prof. A. Kornberg). The reaction products obtained after incubation of the membrane fraction (lanes *2-4*) and the cytosolic fraction (lanes *5-7*) from SaOS-2 cells or synovial fluid from human (lanes *8-10*) for 2 h (lanes *2*, *5*, and *8*), 4 h (lanes *3*, *6*, and *9*), and 12 h (lanes *4*, *7*, and *10*) were analyzed by electrophoresis on urea/polyacrylamide gel followed by autoradiography. P_i, orthophosphate; arrow and dots, polyP of intermediate chain lengths.
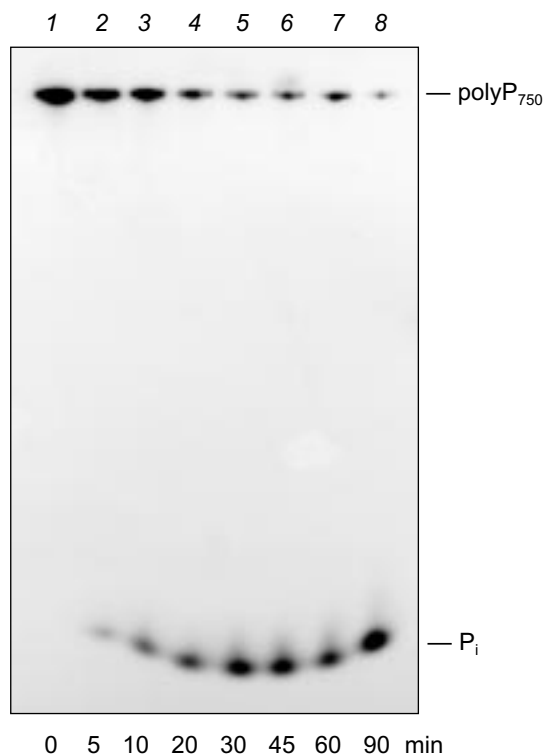
Fig. 5. Degradation of long-chain polyP by purified alkaline phosphatase (calf intestine). Reaction products obtained after incubation of $[^{32}P]polyP_{750}$ in 50 mM Tris-HCl buffer (pH 7.8) at 22°C for different time periods (0 (*1*), 5 (*2*), 10 (*3*), 20 (*4*), 30 (*5*), 45 (*6*), 60 (*7*), 90 min (*8*)) were analyzed on urea/polyacrylamide gel.

EFFECT OF BISPHOSPHONATES USED IN THERAPY OF BONE DISEASES ON PolyP METABOLISM

Bisphosphonates are synthetic analogs of PP_i containing a P–C–P bond instead of P–O–P bond [44]. These molecules are stable under physiological conditions and cannot be degraded by pyrophosphatase, alkaline phosphatase or exopolyphosphatase. Bisphosphonates are effective inhibitors of bone resorption. They are clinically used in the treatment of disorders characterized by excessive bone loss, including osteoporosis, Paget's disease, tumor-induced hypercalcemia, and metastatic bone disease [45]. Bisphosphonates bind to hydroxyapatite crystals and inhibit, like PP_i and polyP, both precipitation and dissolution of calcium phosphate [46-48].

We found that osteoblast exopolyphosphatase activity is inhibited by the bisphosphonates, etidronate and, to a lesser extent, clodronate and pamidronate [10]. Etidronate inhibits the degradation of both long and short polyP chains by the exopolyphosphatase [10]. These results indicate that polyP metabolism in bone tissue may be a further target for these drugs.

However, it should be noted that more than one mechanism is most likely involved in the mode of action of bisphosphonates. These drugs inhibit the activity, recruitment, and adhesion of the bone-resorbing osteoclasts and reduce osteoclast lifespan [49].

PolyP IN DENTISTRY

PolyP is often used as a component of toothpaste. It has been shown to possess anticaries activity [50]. This effect of polyP can be explained by two mechanisms: polyP can reduce both acid solubility [23, 51, 52] and mineralization of hydroxyapatite [53]. Topical administration of bisphosphonates decreases formation of dental calculus. Bisphosphonates were also found to inhibit experimental periodontitis characterized by excessive bone resorption in rats and monkeys [54-56].

CONCLUSION AND FUTURE ASPECTS

Our studies suggest that besides PP_i, polyP is involved in the mineralization process in bone tissue. This assumption is supported by the following findings: 1) in osteoblasts higher concentrations of polyP exist than in other mammalian cells [10]; 2) long-chain polyP molecules inhibit hydroxyapatite precipitation *in vitro* stronger than PP_i [22]; 3) polyP also affects mineralization in cultured cells; 4) osteoblasts possess comparably high polyP-degrading exopolyphosphatase activity [10]; 5) this enzyme can eliminate the mineralization inhibitor polyP allowing mineralization to occur. Moreover, P_i required for calcium precipitation is generated during the enzyme reaction; 6) in osteoblasts, exopolyphosphatase activity and polyP content respond to modulators of osteoblast growth and function [10]; and 7) exopolyphosphatase is inhibited by bisphosphonates [10], indicating that polyP metabolism in bone tissue represents a further target for these drugs.

However, there are a number of questions, which should be addressed in future studies, using the osteoblast model. These studies should include more detailled investigations of the effects of modulators of osteoblast proliferation/differentiation on polyP metabolism. In particular, it might be interesting to investigate both the independent effects and the combined effects of modulators. For example, it is known that $1,25(OH)_2D_3$ increases the expression of the osteocalcin gene, whereas dexamethasone suppresses the expression of this gene. However, the combination of both agents causes a synergistic effect.

During osteoblast development, a sequential expression of genes occurs, possibly including genes for polyP-dependent enzymes. Therefore, the effect of some modulators on polyP metabolism could depend on the

specific phase of osteoblast differentiation. Osteoblasts may also represent a suitable model to investigate the role of polyP in apoptosis due to their high polyP content [14]. Apoptotic osteoblasts are observed in heavily mineralized cultures [57].

Further studies have to clarify the intracellular localization of polyP and polyP-dependent enzymes in osteoblasts. It is not yet known whether polyP molecules are present in a free state or associated with some other cellular components in these cells.

One important question concerns the mechanism of formation of extracellular polyP in bone tissue. PolyP might be released into the extracellular space by osteoblasts or synthesized extracellularly. It might be interesting to investigate the effect of polyP on dissolution of the organic bone matrix (polyP is an inhibitor of cathepsin) and the effect of polyP binding to collagen fibrils on the mineralization process. Colla-genases and cathepsin are the most important catabolic enzymes of the organic bone matrix; cathepsin is inhibited by polyP [58].

Furthermore, polyP might influence the signal transduction processes in osteoblasts. Thus, it is interesting to investigate the role of polyP in regulation of intracellular free calcium concentration and intracellular pH in osteoblasts. PolyP has been shown to act as a modulator of enzymes involved in signal transduction; e.g., polyP is able to stimulate the rate of formation of the *ras* protein–GTP complexes (regeneration of the GTP form from the GDP form) [59]. PolyP is an inhibitor of the cAMP phosphodiesterase [60] and phospholipase A_2 [61].

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