

RESEARCH ARTICLE

Amorphous Ca²⁺ polyphosphate nanoparticles regulate the ATP level in bone-like SaOS-2 cells

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ABSTRACT

Polyphosphate (polyP) is a physiologically occurring polyanion that is synthesized especially in bone-forming osteoblast cells and blood platelets. We used amorphous polyP nanoparticles, complexed with Ca²⁺, that have a globular size of ~100 nm. Because polyP comprises inorganic orthophosphate units that are linked together through high-energy phosphoanhydride bonds, we questioned whether the observed morphogenetic effect, elicited by polyP, is correlated with the energy-generating machinery within the cells. We show that exposure of SaOS-2 osteoblast-like cells to polyP results in a strong accumulation of mitochondria and a parallel translocation of the polyP-degrading enzyme alkaline phosphatase to the cell surface. If SaOS-2 cells are activated by the mineralization activation cocktail (comprising β-glycerophosphate, ascorbic acid and dexamethasone) and additionally incubated with polyP, a tenfold intracellular increase of the ATP level occurs. Even more, in those cells, an intensified release of ATP into the extracellular space is also seen. We propose and conclude that polyP acts as metabolic fuel after the hydrolytic cleavage of the phosphoanhydride linkages, which contributes to hydroxyapatite formation on the plasma membranes of osteoblasts.

KEY WORDS: Polyphosphate, Bone formation, SaOS-2 cells, ATP level, Regenerative medicine, Tissue engineering

INTRODUCTION

In addition to DNA and RNA, a third polyanion, polyphosphate (polyP), is present in bacteria, archaeobacteria and all multicellular organisms – plants, fungi and animals (Lohmann, 1928; Langen and Liss, 1958; Kornberg et al., 1999; Schröder and Müller, 1999; Schröder et al., 2000; Kulaev et al., 2004). Its chemical structure is very simple and comprises linearly arranged orthophosphate units (P_i) that are linked through high-energy phosphoanhydride bonds. In a physiological-pH environment, each internal P_i of the polymer exposes a monovalent negative charge, rendering polyP as an intensely anionic polymer. With cations, polyP forms salts (Fig. 1). The chain lengths of the polymer vary between a few phosphates to several thousand units (Kornberg et al., 1999). In bacteria, polyP is synthesized enzymatically from ATP in a fully reversible way; hence, it has been proposed that bacteria are able to form ATP from

intracellularly deposited polyP (Kuroda and Kornberg, 1997; Kulaev et al., 2004). In turn, a series of enzymes have been discovered and characterized that catalyze the catabolic and anabolic pathways of polyP in bacteria, whereas only a few had been identified in Metazoa (reviewed in Rao et al., 2009; Lorenz et al., 1994). PolyP exists in different chain-size classes in organisms. The long-chain polyPs, with more than 100 phosphate units (polyP>100), are present in infectious microorganisms; medium-chain polyphosphates (polyP≈40) are secreted from activated human platelets and might modulate blood clotting (Morrissey et al., 2012; Faxälv et al., 2013). In addition, high levels of polyP have also been reported in myeloma cells (Jimenez-Núñez et al., 2012), as well as in cells of the heart, brain and kidney (Kulaev et al., 2004). The level of polyP in human osteoblasts, platelets and blood plasma is especially high (Schröder et al., 2000). Recently, considerable amounts of polyP have been isolated from human osteoblast-like SaOS-2 cells (Khong and Tanner, 2014). In mammals, the alkaline phosphatase (ALP; EC 3.1.3.1) has been identified as a potent exopolyphosphatase that comprises a decreasing affinity (Michaelis–Menten, K_m) constant for polyP with increasing chain length (Lorenz and Schröder, 2001). In addition, the existence of endopolyphosphatases has been analytically proven, but they have not yet been purified (Müller et al., 2015a). The released orthophosphate can act, similar to inorganic phosphate, as a source for the mineralization process of cells in culture (Wiens et al., 2010b; Müller et al., 2011); likewise, the orthophosphate can inhibit carbonic anhydrase, the seed-forming enzyme during bone mineral deposition *in vitro* (Müller et al., 2013). Inorganic phosphate can replace β-glycerophosphate in the mineralization-activation cocktail (MAC, usually comprising β-glycerophosphate, ascorbic acid and dexamethasone) *in vitro* (Müller et al., 2011).

Since the initial studies, which used primary cultures of human osteoblast-like cells that had been prepared from human mandible biopsy material (Fleisch et al., 1966; Leyhausen et al., 1998), and those that occurred later using cell lines, such as SaOS-2 cells, the anabolic effect of polyP on bone cell mineralization has been demonstrated (Hacchou et al., 2007; Usui et al., 2010; Müller et al., 2011; Wang et al., 2013; and reviewed in Wang et al., 2014a). One prerequisite for this activating function of polyP is that the soluble Na⁺ salt of polyP (Na-polyP) is complexed with Ca²⁺ in order to prevent Ca²⁺ deprivation in the medium (Müller et al., 2011). Very recently, a compelling report has proposed that the metabolic effect of polyP might be dependent on the morphology of the polyP Ca²⁺ salt (Ca-polyP) polymer added (Ariganello et al., 2014). A solution to those diverging results came after the introduction of the standardized protocol for the preparation of Ca-polyP (Müller et al., 2015a). Emphasis had been put on the development of polyP material that remains amorphous, is of nanosized morphology and comprises a size dimension of 100–200 nm that is suitable for cellular uptake (Voigt et al., 2014). Previously, polyP that was integrated into inorganic

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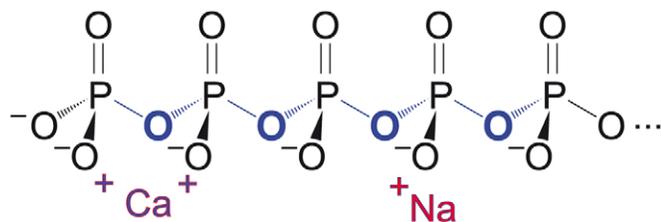


Fig. 1. Structure of linear polyP. The orthophosphate (P_i) units are linked together, after condensation, through high-energy phosphate bonds (pyrophosphate, acid anhydride linkages; blue). The negatively charged P_i groups can bind mono- (Na⁺), divalent- (Ca²⁺) or polyvalent cations (red).

matrices had been sintered, resulting in larger particles with a highly edged morphology (Pilliar et al., 2001; Ding et al., 2008). Using these nanosized Ca-polyP, as prepared here, it has been demonstrated that this polymer elicits morphogenetic activity from SaOS-2 cells, displays a hardness of ≈ 1.3 GPa, a value that is between that of bone and cartilage (Wang et al., 2014), and is prone to hydrolytic degradation during *in vitro* incubation of the cells (Müller et al., 2015a).

Having compelling evidence that Ca-polyP, fabricated in the form of amorphous Ca-polyP nanoparticles, termed aCa-polyP-NP, acts on SaOS-2 cells morphogenetically and is prone to hydrolytic degradation through exo- and endohydrolytic cleavage of the energy-rich bonds, the resulting question regarding the fate and potential metabolic utilization of the released Gibbs free energy has to be addressed. The data presented here demonstrate that, after exposure of SaOS-2 cells to aCa-polyP-NP, they respond with an accumulation of mitochondria, an increased intracellular level of ATP and an intensified release of ATP into the extracellular milieu. These results indicate that polyP increases the energy-generating metabolism in the mitochondria of bone cells.

RESULTS

The polyP sample

Amorphous Ca-polyP nanospheres were prepared by precisely following a previously described protocol (Müller et al., 2015a). It is crucial that the addition of CaCO₃ to the Na-polyP solution is performed drop wise under a constantly controlled pH of 10.0. At the stoichiometric ratio of 1:2 (phosphorous to calcium), a powder of amorphous Ca-polyP is obtained, termed aCa-polyP-NP, that comprises 50- to 130-nm large spheres (Fig. 2A,B).

Mitochondria abundance increases in SaOS-2 cells after incubation with aCa-polyP-NP

The SaOS-2 cells were incubated as described in Materials and Methods. At first, the cells were incubated for a period of 3 days in the absence of the MAC, the MAC was then added and the cells were incubated for a further 4 days in the absence or presence of 30 $\mu\text{g/ml}$ aCa-polyP-NP. Subsequently, the cells were harvested,

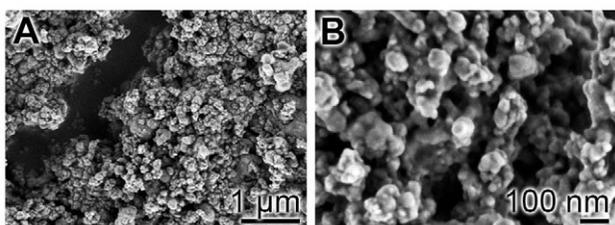


Fig. 2. Morphology of aCa-polyP-NP used in the present study. (A,B) Scanning electron microscopy images at different scales.

sliced and analyzed by using transmission electron microscopy (Fig. 3A,B,D,E). The thin sections revealed that the lobulated osteoblast-like SaOS-2 cells (Baxter et al., 2002) from untreated cells contained only a low number of mitochondria (Fig. 3A,B), whereas those cells that had been exposed to aCa-polyP-NP contained dense clusters of mitochondria (Fig. 3D,E). The overall cell morphology was identical in both cultures.

Translocation of the alkaline phosphatase in response to polyP

The ALP is a marker protein for mature osteoblasts (see Wang et al., 2012). In addition, it has been described that the enzyme has an altered pattern of glycosylation during bone disease development, which is dependent upon the intra- and extracellular localization of the enzyme (Langlois et al., 1994). Furthermore, the results of light-microscopy analyses, used to localize the ALP through enzyme histochemistry, indicate that the ALP translocates to the membrane region after activation of the osteoblasts (Nakano et al., 2004).

To elucidate whether the enzyme changes its localization after exposure to aCa-polyP-NP, immunogold staining and TEM-based analyses were performed in order to locate the enzyme. Again, cells had been incubated for an initial period of 3 days, and then cultured for 3 days in the presence of MAC, additionally either in the absence (Fig. 3C) or presence of 10 $\mu\text{g/ml}$ aCa-polyP-NP (Fig. 3F). After immunogold labeling, the ALP molecules in cells that had not been treated with the nanoparticles were present in a scattered pattern and were not seen frequently (Fig. 3C). In contrast, the enzyme in cells that had been exposed to aCa-polyP-NP were abundantly, and almost exclusively, detected in association with the cell membrane (Fig. 3F). Very often, the labeled molecules appeared in clusters. In the controls, when the antibodies were omitted from the staining procedure, no clusters of gold were detected (data not shown).

Effect of aCa-polyP-NP on the intracellular ATP level

The basal levels of ATP (time 0 of incubation) within SaOS-2 cells, in untreated (without MAC) and in MAC-treated cells was ≈ 0.6 pmol/ 10^3 cells (Fig. 4A,B). However, when the untreated cells were exposed to 30 $\mu\text{g/ml}$ of aCa-polyP-NP for 3 days, a significant increase to 1.25 pmol/ 10^3 cells was measured, whereas all other values that had been determined at day 1 or after 3 days of treatment with lower concentrations of aCa-polyP-NP only increased insignificantly (Fig. 4A). By contrast, SaOS-2 cells that had been exposed both to MAC and aCa-polyP-NP responded with a significant increase of the ATP level only 1 day after the addition of 10 $\mu\text{g/ml}$ or 30 $\mu\text{g/ml}$ of aCa-polyP-NP (Fig. 4B). The highest ATP concentrations were determined 3 days after the addition of the polymer with 1.3 pmol/ 10^3 cells (at 10 $\mu\text{g/ml}$) and 2.1 pmol/ 10^3 cells (30 $\mu\text{g/ml}$). The lower concentration of aCa-polyP-NP (3 $\mu\text{g/ml}$) did not cause any significant effect in SaOS-2 cells, irrespective of the presence of MAC.

ATP release is increased after incubation with aCa-polyP-NP

The release of ATP from the cells had been determined by quantification of the nucleotide level in the medium, without serum, after an incubation period of 30 min. The basal level of ATP release from cells, incubated in the absence or presence of MAC, at time 0, was low at a concentration of 0.3 fmol/ 10^5 cells (Fig. 4C,D). In cultures that remained untreated with respect to MAC, the extent of ATP release during the 30-min incubation period increased significantly if the cells were incubated for 3 days in the presence of 10 $\mu\text{g/ml}$ or 30 $\mu\text{g/ml}$ of the polymer (Fig. 4C). Prolonging the incubation period to a total of 5 days resulted in a significant increase in ATP release upon treatment with each of the polymer

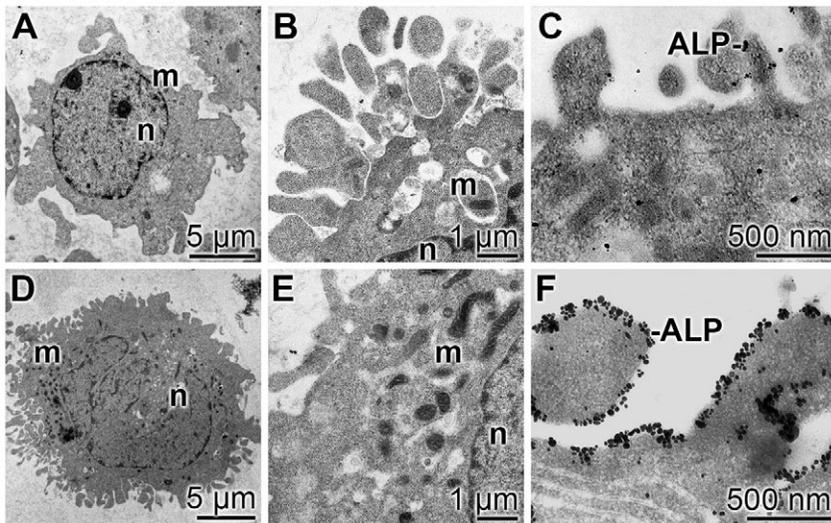


Fig. 3. Abundance of mitochondria in SaOS-2 cells, and aCa-polyP-NP-dependent localization of the ALP. The cells were treated for 3 days with the MAC in the absence of aCa-polyP-NP (A–C) or the presence of 30 µg/ml aCa-polyP-NP (D–F). Transmission electron microscopy images of immunogold labelling are shown. The abundance of mitochondria, 'm', within SaOS-2 cells that had not been treated with polyP was low (A,B), whereas mitochondria were abundant in cells that had been exposed to aCa-polyP-NP for 3 days (D,E). n, nucleus. Also, the localization of the ALP was drastically different – (C) in untreated cells, the enzyme was present in a scattered pattern within the cells, whereas almost all ALP-gold-intensified immunocomplexes were clustered at the surface of cells that had been exposed to aCa-polyP-NP (F).

concentrations used (3 µg/ml, 10 µg/ml and 30 µg/ml). However, if the cells had been additionally incubated with the MAC and aCa-polyP-NP, ATP release was much higher for cells that had been incubated with the polymer for 3 or 5 days (Fig. 4D). The highest ATP release (3.8 fmol/10⁵ cells) was measured after incubation with 30 µg/ml of the polymer for 5 days.

DISCUSSION

Any biological or biochemical system is characterized by relatively stable non-equilibrium steady-state forms that follow the thermodynamic laws. This basic principle is not only applicable to bio-organic reactions, e.g. metabolic cycles, but also for

bio-inorganic reactions, e.g. mineral deposition in bone. The maintenance of a biological or biochemical non-equilibrium requires continuous chemical energy input that sustains this state while the concentrations of the substrates and/or the products fluctuate owing to the metabolic reactions that are coupled to them. Focusing on bone mineral deposition, structural and functional molecules on which biomineralization can proceed are prerequisites for this process, as well as key molecules that drive and facilitate the reactions, such as enzymes and ion channels, and lower the activation energy that exists as an energy barrier before an exergonic reaction can run.

Collagen fibrils are the structural molecules onto which >80% of the mineral in mammalian bone is deposited (Wassen et al., 2000).

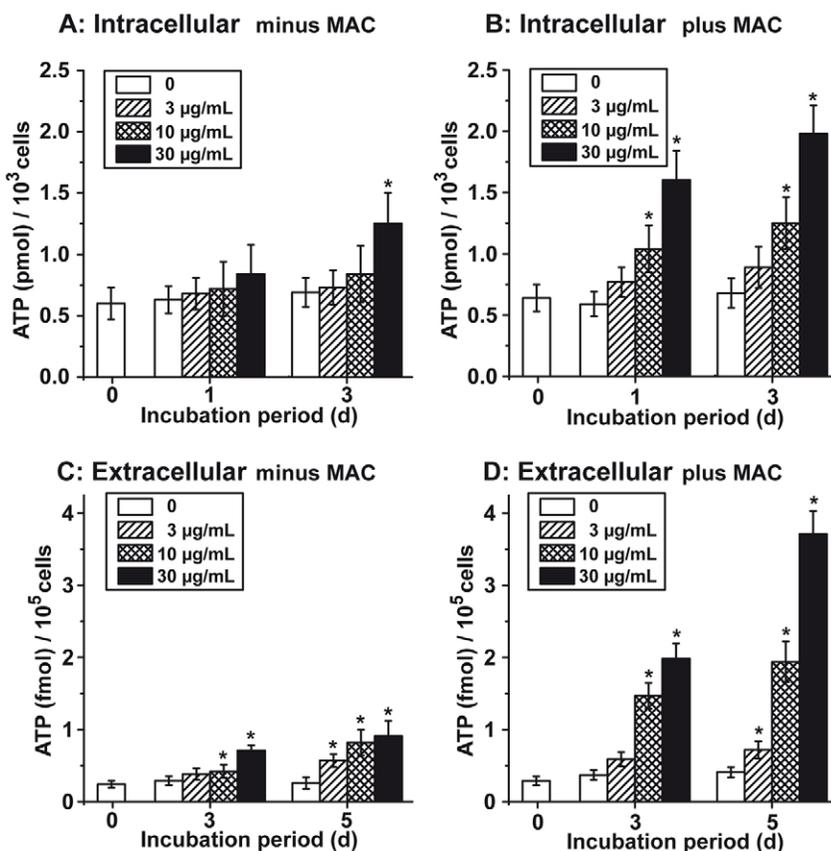


Fig. 4. The intracellular ATP level and the efficiency of ATP release in SaOS-2 cells. The cells remained either untreated (A,C) or treated (B,D) with respect to MAC. (A,B) Intracellular and (C,D) extracellular ATP levels were determined as described in Materials and Methods by using the ATP luminescence method (luciferin-luciferase assay). Three concentrations of aCa-polyP-NP were used – 3 µg/ml, 10 µg/ml and 30 µg/ml. Values are means ± s.e.m. (n=10); significant differences to the controls (0 µg/ml of the polymer) are marked. *P<0.01. d, days.

On those fibrils, the functional groups that promote mineral deposition are the hydroxylysine and the hydroxyproline groups, together with the glycosylation sites. The functionalization of these sites is driven by enzymes – lysyl hydroxylase, prolyl 4-hydroxylase and glycosyl transferases – subsequently providing the platform for calcification, involving the deposition of the calcium-based salts and crystals. The deposition processes that result in the formation of hydroxyapatite, perhaps through calcium carbonate (CaCO_3), are exergonic (Wang et al., 2014b). Besides phosphorus (as P; around 15.2 wt%), carbonate (as CO_3^{2-}) also exists in human bone to 7.4 wt% (Dorozhkin, 2011). The carbonic anhydrase enzyme(s) are likely to be involved in the formation of CaCO_3 , acting as bioseeds, whereas the ALPs provide the building units for hydroxyapatite formation – P_i and Ca^{2+} – thereby facilitating the process of mineralization at body temperature (Wang et al., 2014a). The transport of those components, e.g. P_i , require special transporters, such as the type III sodium-dependent phosphate transporter in osteoblasts (Nielsen et al., 2001). In turn, homeostasis of phosphate is crucially dependent on the cellular ATP synthesis, as shown previously for this transporter, which has been proven to play an important role during bone mineralization (Sugita et al., 2011).

In the present study, we have used a nanoparticle preparation of Ca-polyP that has an amorphous state and a globular nanosphere morphology (Müller et al., 2015a). The polyP remains in the nanoparticles in the polymeric form; from previous analyses, it is known that the chain lengths of polyP in those nanoparticles remains around 40 units (Müller et al., 2015a). There were no indications of the presence of pyrophosphate, which has been previously found to inhibit bone mineral formation *in vitro* (Addison et al., 2007). The nanoparticles are taken up by the cells through clathrin-mediated endocytosis (Müller et al., 2015b) because the process can be inhibited by the specific inhibitor trifluoromazine (Chen et al., 2009). This form of particle uptake through clathrin-mediated endocytosis is abundantly seen in osteoblasts (Ellington et al., 1999). During the exposure of SaOS-2 cells to polyP a distinct translocation of the ALP from the cytoplasm to the cell membrane occurs, a process that strongly indicates that ALP is functionally active during phosphate deposition into the hydroxyapatite crystals (Magnusson et al., 1999). This result supports earlier evidence that the ALP can associate with the plasma membrane of simian cells (Berger et al., 1987).

Until now, no clear evidence has been presented that could explain where the ATP originates from during osteogenesis. Also, the potential involvement of the hormones leptin and insulin gives only an answer on the involvement of the regulatory molecules (Long, 2012; Turner et al., 2013). Compelling evidence is available that favors the concept that polyPs are polymers that participate in bone formation (Tsutsumi et al., 2014; reviewed in Wang et al., 2014a). Therefore, it was pressing to investigate whether polyP, with its energy-rich phosphate anhydride linkages, can serve as a free-energy donor. It is very evident that the free-energy change ($\Delta G^\circ = -30.5$ kJ/mol), liberated during enzymatic hydrolysis of the anhydride linkages by exo- (ALP) and/or endo-phosphatases, must be removed from the tissue or re-used in metabolic cycles. It has been calculated that human blood – specifically only in blood platelets, which are the major reservoir for polyP in humans – contains 1–3 μM polyP50 (polyP with a chain length of 50 phosphate units) after full platelet activation (Morrissey et al., 2012). This finding implies that 25 mg of polyP50 is present in all blood platelets.

The first indication that the free-energy that is conserved in the anhydride linkages of polyP is connected with the biochemical and/or metabolic status of the polyP-exposed cells has been recently published (Tsutsumi et al., 2014). The data obtained by using

murine MC3T3-E1 osteoblastic cells reveals that, after exposure of those cells to polyP, a change in the cell morphology occurs as the result of the formation of a well-developed Golgi, the activation of the rough endoplasmic reticulum, as well as the appearance of large mitochondria. In our study, we have demonstrated and observed that SaOS-2 cells that have been exposed to polyP drastically increase their number of mitochondria; an estimation of the increase reveals a fivefold accumulation. Because the number of mitochondria correlates with the amount of ATP produced in them (Gajewski et al., 2003), we determined the effect of polyP on the intracellular ATP level in both untreated and MAC-treated cells. The results were surprisingly significant. In those cells that had been activated with the MAC components, which accelerate mineralization in bone and bone-like cells (Park, 2012), a significant (approximately tenfold) increase in the ATP level was measured. From this finding we have to draw the conclusion that osteoblasts synthesize high levels of ATP, not only to activate the organic metabolism during maturation of these cells but also to allow the mineral deposits to form.

Extracellular ATP levels control and modulate the activity of both the osteoblasts (Orriss et al., 2013) and the osteoclasts (Miyazaki et al., 2012), implying that the nucleotides released by osteoblasts in bone could locally affect mineralization. By contrast, *in vitro*

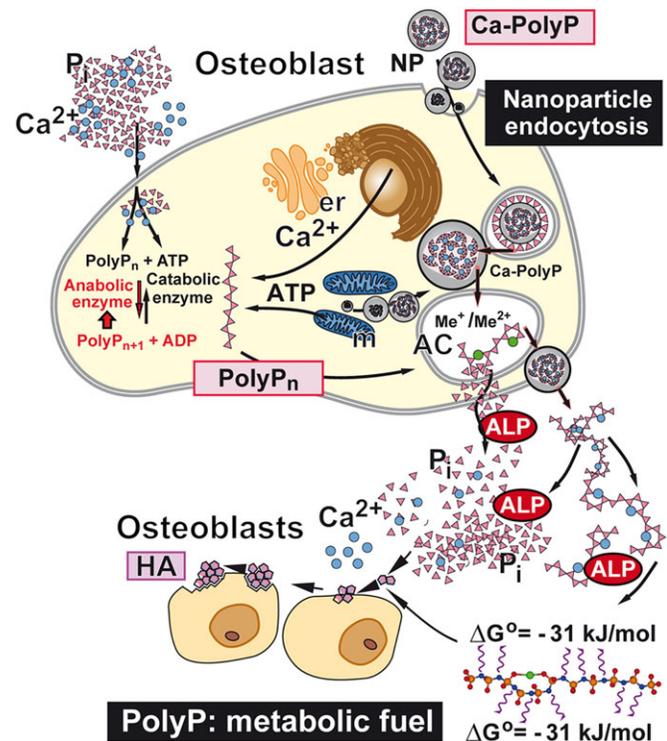


Fig. 5. Schematic outline of the proposed role of polyP during biomineralization of bone cells. Both P_i and Ca^{2+} are taken up by cells through special transporters, e.g. P_i by the Na-dependent phosphate transporter. Intracellularly, polyP is formed by hypothesized anabolic enzymes and, perhaps, (to a lesser extent) degraded again by catabolic phosphatases. The required ATP for polyP is generated in mitochondria (m), whereas Ca^{2+} is released from endoplasmic reticulum (ER). However, the Ca-polyP nanoparticles, used in the present study, are taken up through clathrin-mediated endocytosis and presumably released into the extracellular space after storage in acidocalcisomes (AC). polyP can form salts with Na^+ , Ca^{2+} or other mono (Me^+) or divalent (Me^{2+}) cations. In the extracellular space polyP can function as metabolic fuel during phosphatase-mediated hydrolysis (e.g. ALP). On the extracellular side of the plasma membrane, the hydroxyapatite (HA) crystals are deposited.

experimental evidence reveals that ATP, but not ADP, promotes the synthesis of the ALP, as well as of the bone morphogenetic proteins (BMP)-2, BMP-4 and BMP-5 (Ayala-Peña et al., 2013). Again, exposure of the SaOS-2 cells to polyP revealed a strong (tenfold) release of ATP from MAC-treated cells.

Taken together, the data presented here show that polyP in the SaOS-2-cell system increases the number of mitochondria and the ATP content within the osteoblast-like cells, as well as the release of this nucleotide after exposure to polyP. A scheme is proposed that might explain the route of polyP formation and the effect of this polymer on mitochondria, as well as its potential role as ‘metabolic fuel’ to be consumed during mineral deposition (Fig. 5). Future studies should now follow that study the role of ATP during hydroxyapatite formation in more detail, e.g. incubation of osteoblasts under high ATP concentrations in the medium or under conditions of ATP deprivation. An understanding of the direct effects of the hormones leptin and insulin during mineralization are also lacking.

MATERIALS AND METHODS

Materials

Na-polyP with an average chain length of ~40 phosphate units was obtained from Chemische Fabrik Budenheim (Budenheim, Germany).

Preparation of amorphous Ca-polyP nanospheres

aCa-polyP-NP was prepared as described previously (Müller et al., 2015a). Briefly, 10 g of Na-polyP was dissolved in distilled water, then 28 g of CaCl₂ in distilled water was added drop wise to the Na-polyP at room temperature. During the complete procedure, the pH had been adjusted to 10.0 with a NaOH aqueous solution. After stirring for 4 h, the particles were collected, washed twice with ethanol and dried at 60°C. Fourier transform infrared spectroscopy was applied to verify the polymer characteristics of the nanoparticles; X-ray diffraction analysis demonstrated that the material was amorphous (Müller et al., 2015a).

Cultivation of SaOS-2 cells

SaOS-2 cells (human osteogenic sarcoma cells) were cultured in McCoy’s medium (Biochrom-Seromed, Berlin, Germany) that had been supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum (FCS), and 100 units/ml penicillin and 100 µg/ml streptomycin (Wiens et al., 2010a). The cells were incubated in 25-cm² flasks or in 6-well plates (surface area 9.46 cm²; Orange Scientifique, Braine-l’Alleud, Belgium) in a humidified incubator at 37°C. The cultures were started with 3 × 10⁴ cells/well in a total volume of 3 ml. After an initial incubation period of 3 days, the cultures were continued to be incubated for a total of 5 days in the absence or presence of the MAC, comprising 5 mM β-glycerophosphate, 50 mM ascorbic acid and 10 nM dexamethasone to induce biomineralization (Wiens et al., 2010b). The polyP sample, aCa-polyP-NP, was added to the cultures at concentrations between 0 and 30 µg/mL, as indicated.

Electron microscopy

For the scanning electron microscopy analyses, a HITACHI SU 8000 electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) was employed.

The methods and techniques applied for transmission electron microscopy analyses has been recently published (Kokkinopoulou et al., 2014). The cells were fixed in paraformaldehyde and glutaraldehyde, embedded in agarose and then in LR-white resin (no. 62661, Sigma-Aldrich). After polymerization, ultrathin sections (80 nm) were cut (Microsystems, Wetzlar, Germany). For immunogold labeling in transmission electron microscopy analyses, the cells were incubated with a monoclonal IgG antibody against human alkaline phosphatase (produced in mouse, no. A2064, Sigma-Aldrich) and then with a secondary anti-mouse antibody coupled to 10-nm gold particles (1:50 diluted with water,

Sigma-Aldrich). The samples were enhanced with silver (Danscher, 1981), and contrasted with uranyl acetate and lead citrate. The slices were inspected with a TemCam-F416 (4K×4K) CCD camera (TVIPS, Gauting, Germany) operated on a Tecnai 12 transmission electron microscope (FEI, Eindhoven, The Netherlands) at an accelerating voltage of 120 kV. In the controls, the antibodies were omitted during the procedure.

Determination of intracellular ATP level

ATP was extracted from SaOS-2 cells (Stanley, 1986; Moriwaki et al., 2013), and the concentration was subsequently determined by using the ATP luminescence kit (no. LL-100-1, Kinshiro, Toyo Ink, Japan). After establishing a standard curve for given ATP concentrations, the absolute amount of ATP was extrapolated and is given as pmol/10³ cells.

Determination of ATP release

Immediately before the determination of the ATP release, the cells were transferred into medium without FCS and continued to be incubated for an additional 30 min at a cell concentration of 10⁵/ml. Then the samples were frozen until use for determination of ATP in the luciferin-luciferase assay (Orriss et al., 2009). After establishing of the calibration curve, the ATP concentrations in the samples were determined and the values were normalized to 10⁵ cells.

Statistical analysis

The results were statistically evaluated using paired Student’s *t*-test (Sachs, 1984).

Competing interests

The authors declare no competing or financial interests.

Author contributions

E.T., J.S.M. and M.K. performed the experiments. W.E.G.M., X.W., Q.F. and H.C.S. designed the experiments and analyzed the data. W.E.G.M. and X.W. wrote the manuscript.

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