Tatiana Kulakovskaya · Evgeny Pavlov
Elena N. Dedkova *Editors*

Inorganic Polyphosphates in Eukaryotic Cells

Springer

Inorganic Polyphosphates in Eukaryotic Cells

Tatiana Kulakovskaya • Evgeny Pavlov
Elena N. Dedkova
Editors

Inorganic Polyphosphates in Eukaryotic Cells

Springer

Editors
Tatiana Kulakovskaya
Laboratory of Regulation of Biochemical Processes
Skryabin Institute of Biochemistry and Physiology of Microorganisms
Russian Academy of Sciences
Pushchino, Russia

Elena N. Dedkova
Department of Pharmacology
University of California
Davis, CA
USA

Evgeny Pavlov
NYU College of Dentistry
New York, New York
USA

ISBN 978-3-319-41071-5 ISBN 978-3-319-41073-9 (eBook)
DOI 10.1007/978-3-319-41073-9

Library of Congress Control Number: 2016957150

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland
The registered company address is Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Inorganic polyphosphate is a biological polymer made of many orthophosphates linked together by phosphoanhydride bonds similar to ones found in ATP. polyP is a unique macromolecule that can spontaneously form in nonliving nature and can also be enzymatically produced by living organisms. Over the past few decades, a number of exciting hypotheses regarding the role of polyP in nature have been generated. These range from the concept that polyP can be a prebiotic molecule that was at the origin to living organisms to the concept that polyP can serve as a storage of biological fuel alternative to ATP. Despite its discovery over a century ago, the molecular mechanisms of polyP actions in living organisms remain poorly understood. In fact, most of current knowledge about biological roles of polyP comes from the studies of bacterial organisms and lower eukaryotes with very little studies of higher eukaryotes. One of the central unresolved questions in polyP research is about the relationship between its roles in different organisms. Is polyP indeed a "molecular fossil" which has a role in higher organisms that progressively diminished over the evolution, or is polyP a central player in various biochemical processes throughout all kingdoms of life? Although at present it is impossible to definitively answer this question, recent discoveries regarding the roles of polyP support the idea that polyP maintains an important role in all living organisms including humans. This book presents a collection of chapters dedicated to the current research in the field of eukaryotic polyP. It is divided in two sections with the first focusing on polyP function in simple organisms and the second on polyP function in higher organisms. This book is intended to bring together the perspectives of scientists working in various fields of life sciences, which we believe will provide a broad overview of the current state of the field and help to better understand current achievements and challenges.

We would like to express the gratitude to all authors who contributed to this book for their cooperation, help, and patience.

Pushchino, Russia — Tatiana Kulakovskaya
New York, NY, USA — Evgeny Pavlov
Davis, CA, USA — Elena N. Dedkova

Contents

Part I

Lower Eukaryotes

The Role of Inorganic Polyphosphates in Stress Response and Regulation of Enzyme Activities in Yeast

1

Tatiana Kulakovskaya, Lubov Ryasanova, Vladimir Dmitriev, and Anton Zvonarev

Abstract

Inorganic polyphosphates (polyPs) are multifunctional compounds involved in adaptation of microorganisms to stress. In yeast, polyPs serve a reserve of phosphorus that is consumed by cells with phosphate deficiency. Under phosphate excess, polyP biosynthesis regulates the intracellular phosphate concentration. PolyPs accumulate under conditions of growth suppression under nitrogen starvation and heavy metal toxic stress, and also upon the adaptation of yeast to a hydrophobic carbon source. The participation of polyPs in the regulation of enzyme activities is discussed.

1.1 Introduction

Inorganic polyphosphates (polyPs) are linear polymers containing a few to several hundred orthophosphate residues linked by energy-rich phosphoanhydride bonds (Fig. 1.1). Until recently they were considered molecular fossils, ATP precursors in evolution, and a phosphorus storage mechanism in microorganisms. Igor Kulaev, a pioneer in polyP biochemistry, suggested multiple roles for these polymers in the regulation of cellular processes because of the ability to form complexes with metal ions, proteins, and RNA (Kulaev 1979). The interaction of negatively charged polyPs with anionic biopolymers, such as poly-beta hydroxybutyrate (Reusch 1992) and probably RNA (Kulaev 1979), is mediated by divalent metal cations.

T. Kulakovskaya (✉)
Laboratory of Regulation of Biochemical Processes, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia
e-mail: alla@ibpm.pushchino.ru

L. Ryasanova • V. Dmitriev • A. Zvonarev
Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_1

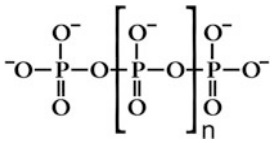

Fig. 1.1 Structure of linear inorganic polyphosphate (polyP)

The excellent studies of Arthur Kornberg and co-workers demonstrated the essential role of polyPs in the switching of gene expression and in cell survival in the stationary phase and under stress (Kornberg 1995; Rao et al. 2009). PolyP and polyphosphate kinase participate in induction of the synthesis of RpoS, an RNA-polymerase subunit in bacteria that is responsible for expression of the genes involved in the stationary phase and adaptation to stress; it is also involved in bacterial cell motility, biofilm formation, and virulence (Rao et al. 2009).

The adaptation of *Escherichia coli* to amino acid starvation is a remarkable example of polyP involvement in the response to stress (Rao et al. 2009). This process is mediated by guanosine 5′-triphosphate, 3′-diphosphate (pppGpp) and guanosine 5′-diphosphate, 3′-diphosphate (ppGpp), the so-called alarmones. These compounds enhance the expression of many stress-induced genes (Magnusson et al. 2005; Sharma and Chatterji 2010). The increase of concentrations of (p)ppGpp in *E. coli* cells enhances the degradation of polyPs by polyphosphatases ppx and gppA (Kuroda 2006). The gppA enzyme also catalyzes the degradation of p(ppGpp) (Keasling et al. 1993). As a result, the polyP content in *E. coli* increases many fold under amino acid starvation. PolyP forms a complex with ATP-dependent Lon proteinase and increases its activity (Kuroda 2006). These polyPs, with an average chain length of 65–700 phosphate residues, bind up to four Lon molecules per one polyP chain, and such a complex is responsible for the proteolysis of ribosomal proteins (Kuroda 2006). The resulting free amino acids are used for biosynthesis of inducible amino acid transporters and enzymes of amino acid biosynthesis.

The mechanisms of polyP participation in stress response in eukaryotic microorganisms are not yet well understood. However, many facts suggest the involvement of polyP in the adaptive mechanisms in fungi:

- The polyP content and chain length are strongly dependent on the growth stage and conditions
- PolyP was found in vacuoles, cytoplasm, cell wall, nuclei, and mitochondria by both subcellular fractionation methods and various microscopic techniques
- The cell compartments possess specific enzymes of polyP metabolism, differently affecting by environmental conditions

This chapter considers changes in polyP content and chain length under various stresses and the involvement of polyP in the regulation of enzyme activities in yeast.

1.2 P_i Limitation and Excess

PolyP content in the cells of *Saccharomyces cerevisiae* strongly depends on the culture medium composition and the growth stage (Vagabov et al. 1998, 2000). The polyP pool of yeast is heterogenic: traditionally five polyP fractions were isolated from *S. cerevisiae*

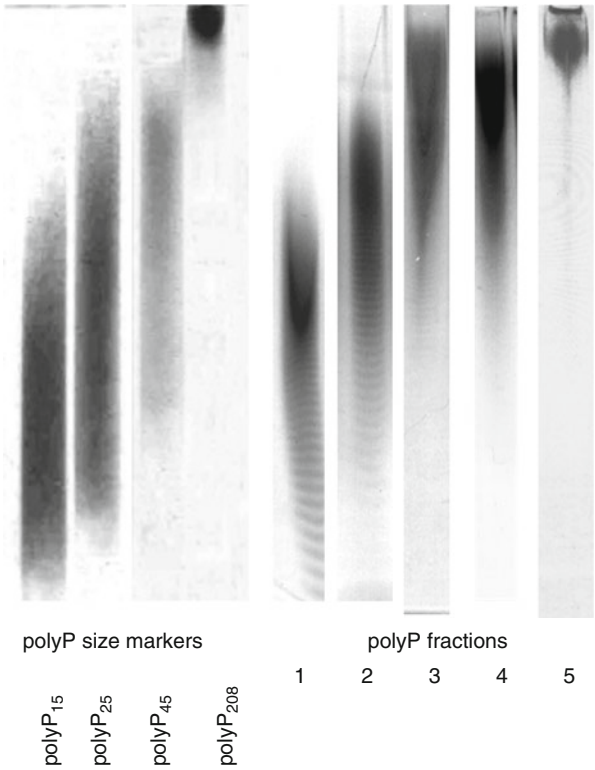

Fig. 1.2 The chain length of polyP fractions of *Saccharomyces cerevisiae* VKM Y-1173 (cultivation in the medium with glucose for stationary stage). PolyP fractions: *1* polyP1, *2* polyP2, *3* polyP3, *4* polyP4, *5* polyP5. PolyP size markers: commercial polyP with average chain lengths of 15, 25, and 45 phosphate residues from Sigma (USA), polyP with average chain length of 208 phosphate residues from Monsanto (USA). The electropherogram of polyP was performed in 24 % polyacrylamide gel with 7 M urea and stained with toluidine blue

cells (Vagabov et al. 1998). The acid-soluble fraction, polyP1, was extracted with 0.5 M $HClO_4$ at 0 °C for 30 min. The salt-soluble fraction, polyP2, was extracted with a saturated solution of $NaClO_4$ at 0 °C for 1 h. The weak alkali-soluble fraction, polyP3, was extracted with weak NaOH, pH 9–10, at 0 °C for 30 min. The alkali-soluble fraction, polyP4, was extracted with 0.05 M NaOH at 0 °C for 30 min. The last fraction, polyP5, was assayed by the amount of P_i, which appeared after the hydrolysis of biomass in 0.5 M $HClO_4$ at 90 °C for 40 min. This fraction was extracted with distilled water (pH 7.0) for 20 h at 0 °C to determine the chain length (Vagabov et al. 2008). The synthesis and degradation of these fractions are closely related to metabolic processes in individual cell compartments; their dynamics is affected differently by changing culture conditions (Vagabov et al. 1998, 2008). These fractions differ in their average chain length (Vagabov et al. 1998, 2000, 2008). A typical PAGE image, demonstrating the chain-length patterns of polyP fractions of *S. cerevisiae*, is shown in Fig. 1.2.

The changes in content of polyP fractions in *S. cerevisiae* cells at different growth stages depend on P_i concentration in the medium (Table 1.1). Under the limit of P_i, the content of polyP in *S. cerevisiae* drops drastically. The so-called ^{31}P-NMR-visible polyPs are exhausted first (Hofeler et al. 1987). With phosphorus deficiency, both PPX1 and PPN1 polyphosphatases are involved in polyP degradation (Lichko et al. 2008). It remains unknown how the hydrolysis of polyP is regulated by phosphate starvation. PPX1 is a constitutive enzyme; however, its activity increased twofold on phosphorus limitation (Kulakovskaya et al. 2004). There are no data on the increase

Table 1.1 The dependence of polyP content in yeast cells on P_i concentration and growth stage

Culture conditions	Time of growth, h	PolyP content, μmol P/g of dry biomass					
		PolyP1	PolyP2	PolyP3	PolyP4	PolyP5	Σ polyP
(–P), early logarithmic stage	4	10	15	6	41	9	81
(–P), late logarithmic stage	8	10	11	20	20	5	66
(+P), early logarithmic stage	4	130	97	55	47	9	338
(+P), late logarithmic stage	8	140	70	40	70	7	327
(+P), phosphate surplus, re-inoculation from (–P) to (+P), early logarithmic stage	4	300	120	250	50	40	760

The wild strain VKM Y-1173 of *S. cerevisiae* was grown in complete Reader medium with 18.3 mM P_i (+P) and phosphate-limited Reader medium with 1.3 mM P_i (–P)

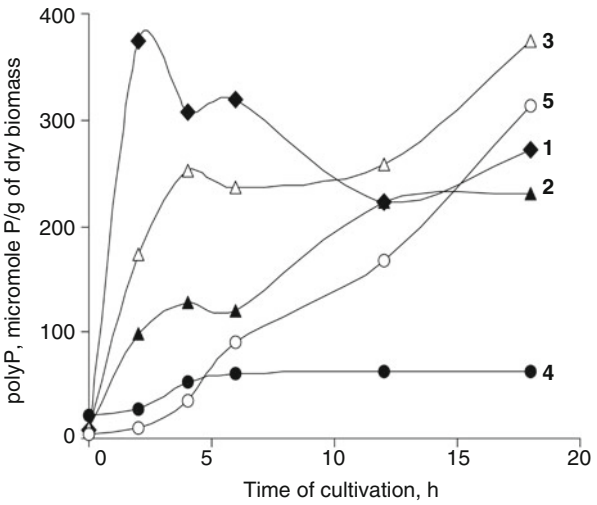

Fig. 1.3 The accumulation of polyP in the cells of *Saccharomyces cerevisiae* VKM Y-1173 at phosphate surplus (Kulakovskaya et al. 2005). The cells grown under P_i limitation were re-inoculated in the glucose-containing Rider medium with 20 mM P_i. PolyP fractions: *1* polyP1, *2* polyP2, *3* polyP3, *4* polyP4, *5* polyP5

in the expression of PPN1 on phosphorus deficiency. It is possible that the ratio of ATP/ADP is one of the regulators of polyP hydrolysis. The endopolyphosphatase activity of PPN1 is suppressed by ATP and stimulated by ADP (Andreeva et al. 2015). The cells of *S. cerevisiae* contain polyP even under conditions of P_i starvation (Table 1.1). It is likely that some polyPs serve as a phosphorus reserve while the others perform regulatory functions. When P_i-starved yeast cells were transferred into complete medium, the polyP content increased sharply (Table 1.1), demonstrating the so-called phosphate surplus (hypercompensation) effect (Liss and Langen 1962). During growth, the levels of polyP fractions changed differently (Fig. 1.3). The content of polyP1, polyP2, and polyP3 increased mainly during the first 2 h. Later, the content of polyP1 decreased and the content of polyP2, polyP3, and polyP5 increased

(Fig. 1.3). The phosphate surplus had no effect on the fraction polyP4: after 4 h, it was restored to the level characteristic for P_i complete medium and remained constant during further cultivation. This suggests the involvement of polyP4 in the cell wall biosynthesis rather than in the maintenance of homeostasis of P_i in the cell (Kulaev et al. 2004). It is difficult to accurately estimate the localization of other polyP fractions, given the heterogeneity of polyP chain length in the cytoplasm, nuclei, vacuoles, and mitochondria (Lichko et al. 2006). The effect of phosphate surplus depends on a cell compartment: it is pronounced in the cytoplasm and mitochondria, but weakly expressed in the vacuoles of *S. cerevisiae* cultivated in the medium with glucose (Trilisenko et al. 2002; Pestov et al. 2004; Andreeva et al. 2008). PolyP is accumulated in mitochondria under glucose repression. In a medium with lactate or ethanol, the quantity of polyP in mitochondria is very low and does not increase with an excess of phosphate in the medium (Pestov et al. 2004; Andreeva et al. 2008). It is probable that besides vtc4, a vacuolar polyphosphate synthase (Hothorn et al. 2009) or other enzymes are responsible for polyP synthesis under phosphate surplus. The *S. cerevisiae* mutants defective in vacuolar ATPase have low levels of polyP. However, under phosphate surplus these mutants accumulated short-chain polyP1 (Tomaschevsky et al. 2010).

In *S. cerevisiae*, conversion of excessive P_i into osmotically inert polyP provides P_i homeostasis in the cell and saves energy in the form of phosphoester bonds.

1.3 Nitrogen Starvation

Under growth suppression by a lack of nitrogen source, the cells of *S. cerevisiae* and *Cryptococcus humicola* accumulated polyP (Breus et al. 2011, 2012). The cells grown at P_i deficiency and containing low levels of polyP (~20 μmol P/g dry weight) were incubated with glucose; and the polyP content, chain length, and localization assessed. PolyP accumulation was stimulated by Mg^{2+}, probably by enhancing P_i uptake via Pho84p, a transporter of yeast plasma membrane that takes up bivalent metal complexes of phosphate (Persson et al. 2003; Reddi et al. 2009). In the presence of Mg^{2+}, the content of long-chain polyP increased in the cells of both species (Table 1.2). The cytoplasm contained numerous small electron-dense inclusions, partly in association with the cytoplasmic membrane and lipid bodies (Fig. 1.4). X-ray microanalysis showed that cytoplasm and the inclusions were rich in phosphorus (Breus et al. 2011). Evidently, polyP accumulation is a mechanism serving to reduce the ATP and P_i levels under conditions of active glycolysis and phosphate excess, when the growth and budding are blocked by the absence of a nitrogen source.

1.4 Toxic Effects of Manganese

An excess of manganese is toxic to many species of yeast: the presence of 2.5–4 mM manganese salts increased the duration of the lag phase, decreased the growth velocity in the log stage, reduced the yield of biomass, and increased the content of polyP (Andreeva et al. 2013, 2014; Ryazanova et al. 2015). The yeasts *S. cerevisiae*

Table 1.2 P_i and polyP content in the cells of *Saccharomyces cerevisiae* and *Cryptococcus humicola* under nitrogen starvation

P_i and polyP content	*Saccharomyces cerevisiae*		*Cryptococcus humicola*	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
P_i, μmol/g dry biomass	90	130	70	70
Total polyP, μmol P/g dry biomass	640	1,450	440	1,200
PolyP*n*,* % of total polyP				
$PolyP_{15}$	58	8	6	5
$PolyP_{45}$	0	0	73	0
$PolyP_{75}$	38	71	0	70
$PolyP_{200}$	4	21	21	25

The cells grown in P_i-limited medium were incubated for 5 h with 30 mM glucose and 5 mM K_2HPO_4 ($-Mg^{2+}$); or with 30 mM glucose, 5 mM, K_2HPO_4, and 5 mM $MgSO_4$ ($+Mg^{2+}$)
**n* is the average number of phosphate residues in the polymer chain

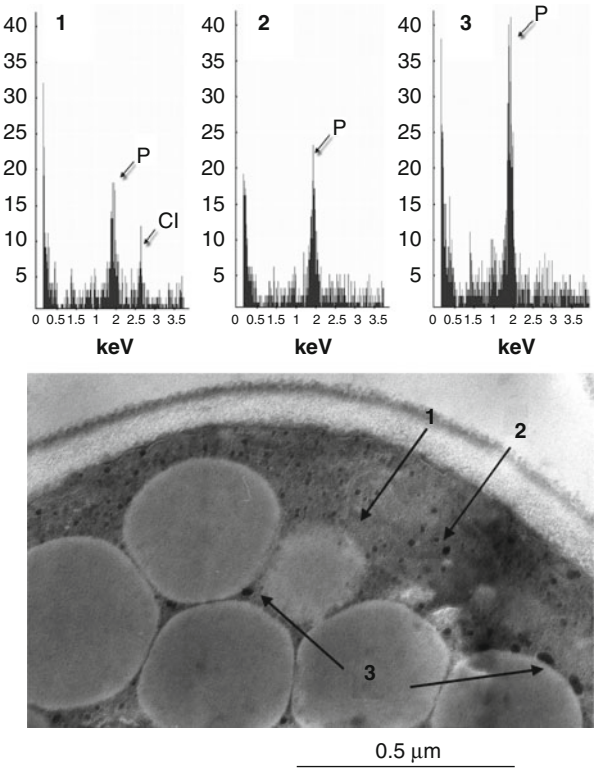

Fig. 1.4 Ultrathin section and X-ray microanalysis of *Saccharomyces cerevisiae* VKM Y-1173 (Breus et al. 2011). The cells were incubated in the medium with 30 mM glucose, 5 mM K_2HPO_4, and 5 mM $MgSO_4$ (see Table 1.2 for polyP content): *1* cytoplasm, *2* electron-dense granules in the cytoplasm, *3* electron-dense granules associated with lipid bodies

(Andreeva et al. 2013) and *Cr. humicola* (Andreeva et al. 2014) demonstrated adaptation to 2.5–4 mM Mn^{2+}, while the growth of *Cryptococcus terreus* was suppressed. Under manganese excess, increased content of the acid-soluble polyP1 was observed in the cells of *S. cerevisiae* (Andreeva et al. 2013) and *Cr. humicola* (Andreeva et al. 2014) (Table 1.3). The cells of *Cr. terreus* did not show such an effect (Table 1.3).

Table 1.3 The effect of Mn^{2+} (2.5–4 mM) on polyP content) in yeast cells

Yeast species	Acid-soluble polyP (μmol/g dry biomass), control/Mn^{2+}	Acid insoluble polyP (μmol/g dry biomass), control/Mn^{2+}
Saccharomyces cerevisiae	66/134	48/44
Cryptococcus humicola	3.5/43	10/30
Cryptococcus terreus	2.9/11	20/43

The cells of *S. cerevisiae* and *Cr. humicola* were grown to the stationary growth stage in the presence and absence of manganese sulfate. Control cells of *Cr. terreus* were grown to stationary growth stage; the time of cultivation in the presence of manganese sulfate was 48 h (no growth was observed)

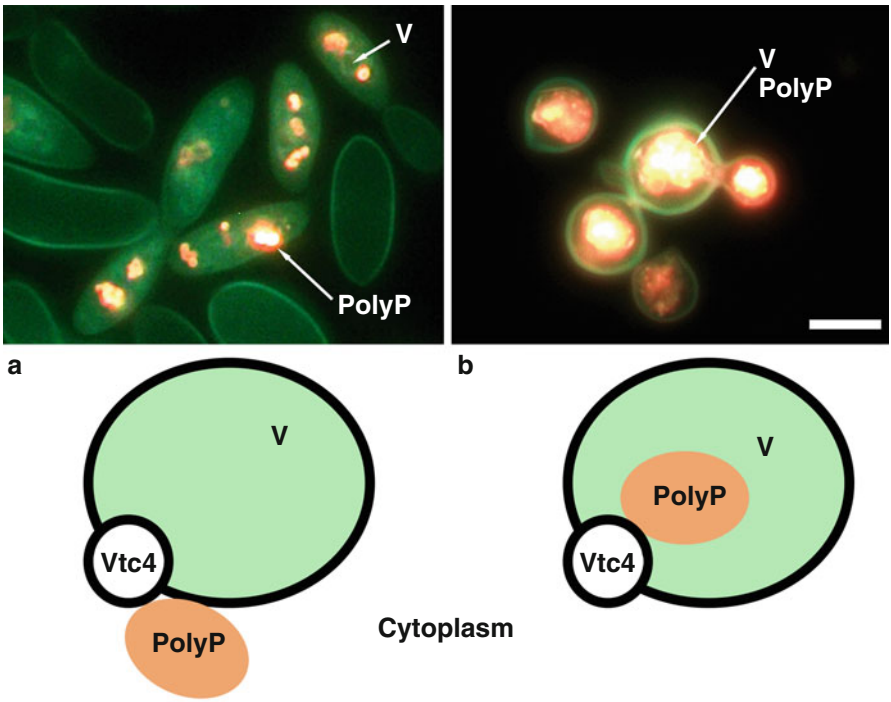

Fig. 1.5 PolyP localization and the hypothetical scheme of polyP accumulation in the cells of *Cryptococcus humicola* (**a**) and *Cryptococcus terreus* (**b**) at manganese excess. The cells were stained by DAPI. Scale bar: 5 μm. *V* vacuole, *Vtc4* polyphosphate synthetase of vacuolar membrane

In *S. cerevisiae*, the average chain length of acid-soluble polyP1 increased in the presence of Mn^{2+} (Andreeva et al. 2013). DAPI fluorescence microscopy showed that in response to the manganese excess, polyP accumulated in cytoplasmic inclusions and the cell wall of *Cr. humicola*, and in vacuoles in the case of *Cr. terreus* (Fig. 1.5). Moreover, in the latter case it changed the morphology of vacuoles and probably disturbed their function. The hypothetical scheme of polyP accumulation in the cells of both species is shown in Fig. 1.5. In *Cr. humicola*, a part of polyP is located close to the vacuolar membrane on the cytoplasmic side, after which the

formed polyP granules are released into the cytoplasm. In *Cr. terreus*, DAPI staining revealed a different situation: polyP accumulated inside vacuoles. The mechanism of vacuolar membrane polyP *s*ynthetase Vtc4 functioning observed in *S. cerevisiae* provides the translocation of the growing polyP chain across the membrane (Hothorn et al. 2009). This suggests the possibility of polyP translocation mainly into the vacuoles or cytoplasm, depending on the peculiarities of Vtc4 proteins in different yeasts. The suggested differences in vtc4 functioning in the cells of *Cr. humicola* and *Cr. terreus* may cause the difference in polyP localization (Andreeva et al. 2014). Accumulation of polyP in cytoplasmic inclusions may be one of the factors providing heavy metal tolerance by forming cation/polyP complexes. On the other hand, polyP is a shunting factor during decreasing growth rate.

1.5 Adaptation to Hydrophobic Carbon Sources

The yeast *Candida maltosa* is capable of using hexadecane as a carbon source. This requires the use of specific enzyme systems and significant structure-function rearrangements in the cell envelope, including the formation of specific enzymatic systems, the so-called lipophilic canals that provide binding and primary oxidation of hexadecane (Dmitriev et al. 2011). Formation of "canals" correlates with the increased activity of cell wall polysaccharide hydrolases β-glucosidase, β-glucanase, and α-mannosidase, and modification of the basic polysaccharides glucan and mannan (Dmitriev et al. 1980). Electron microscopic cytochemistry and immunocytochemistry showed the presence of oxidative enzymes and cytochrome P450 in the canals, and exocellular fibrillar components of yeasts that provided the participation of canals in the primary oxidation of hydrocarbons (Dmitriev et al. 2011). In general, these canals represent a specific molecular complex of the cell wall containing the enzymes responsible for the consumption of hydrocarbons. It is not surprising that the formation of such complexes in the presence of hydrocarbons results in the decrease of the growth rate in comparison with cultivation in a medium with glucose (Table 1.4). The cells grown in the presence of hexadecane accumulated much more long chain polyP (Table 1.4). DAPI fluorescence microscopy

Table 1.4 Growth velocity, P_i and polyP content in the cells of *Candida maltose* at cultivation in the media with glucose or hexadecane

	Carbon source	
Property	Glucose	Hexadecane
Growth velocity, μ/h	1.02	0.58
P_i, μmol/g wet biomass	16	17
PolyP1, μmol/g wet biomass	9.5 (15)	28 (75)
PolyP2, μmol/g wet biomass	7.7 (15)	21 (15)
PolyP3, μmol/g wet biomass	22 (200)	48 (200)
PolyP4, μmol/g wet biomass	8.1 (200)	19 (200)
PolyP5, μmol/g wet biomass	25 (200)	83 (200)
Total polyP, μmol/g wet biomass	72	200

The average number of phosphate residues in the polymer chain of polyP is given in parentheses

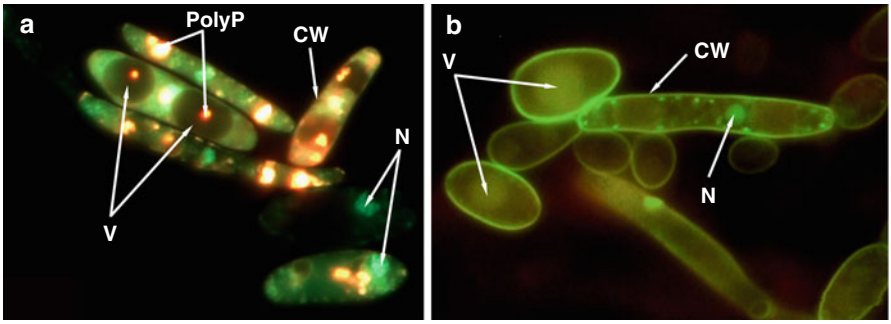

Fig. 1.6 Micrographs of DAPI-stained cells of *Candida maltosa* grown in the medium with hexadecane (**a**) and glucose (**b**); stationary growth stage. Scale bar: 5 μm. *CW* cell wall, *N* nuclei, *V* vacuoles

revealed the presence of polyP in vacuoles, cytoplasm, and cell envelope (Fig. 1.6). In this case it is likely that polyP accumulation in vacuoles and cytoplasm provided the necessary shunting effect at a low growth rate. In the cell wall, polyP apparently plays a specific role in the formation and functioning of "canals."

1.6 PolyP and Regulation of Enzyme Activities

Being a polyanion, polyP can bind with proteins, especially those rich in cationic amino acid residues. PolyP binding proteins were detected in cell extracts of yeast and animals, using a filter binding technique or affinity chromatography on polyP zirconia (Lorenz et al. 1994; Schröder et al. 1999). The effects of polyP on enzymatic activities might involve competition with the polyanionic substrate for the binding site, interaction with polycationic activators or inhibitors, and complexing of cations that influence enzyme activities. In vitro studies revealed that polyP acts as a chaperone; it binds to unfolding high-affinity proteins and supports their refolding (Gray et al. 2014). The long-chain polyP (130 or more phosphate residues on average) was the most effective chaperone (Gray et al. 2014).

PolyP participates in the regulation of activities of some enzymes in yeast cells. PolyP inhibits trehalase from vegetative yeast cells and, to a lesser extent, trehalase from spores (Wolska-Mitaszko 1997), probably because of interactions with polyamines, the activators of the enzyme (App and Holzer 1985). PolyP stimulates the regeneration of the GTP-bound from the GDP-bound form of human and yeast *ras* proteins (De Venditis et al. 1986).

PolyP is essential for the manifestation of the glucan transferase activity of Bg12p, a major protein of *S. cerevisiae* cell wall (Kalebina et al. 2008). Apparently, the role of Bg12p in cell wall consists in the incorporation of newly synthesized mannoprotein molecules, preliminarily bound to glucan chains, into existing glucan carcass. The glucan transferase activity of the obtained enzyme preparation was

determined based on its ability to introduce -1.6-branching into the linear -1.3-glucan. Purified Bg12p had negligible transferase activity with soluble glucan and the glucan isolated from the yeast cell wall. It was suggested, therefore, that glucan transferase Bg12p is a silent cell wall enzyme. However, it became active on incubation with glucan in the presence of polyP with an average chain length of 150 phosphate residues. A hypothetical scheme of the regulation of Bg12p transferase activity by polyP was proposed by Kalebina et al (2008).

The biosynthesis of cell wall mannoproteins and polyP is interrelated (Shabalin et al. 1984; Shabalin and Kulaev 1989). It was supposed that dolichyl-diphosphate:polyP phosphotransferase solubilized from the preparation of transport vesicles is responsible for this interrelation (Shabalin and Kulaev 1989). Dolichol-phosphates (Dol-P) act as transmembrane carriers of carbohydrate residues in glycoprotein biosynthesis. At the cytoplasmic side of the endoplasmic reticulum, GDP-mannose interacts with the phosphate residue of Dol-P. The Dol-P-P-mannose is transferred across the membrane in such a way that the phosphomannose residue enters the lumen, where mannosyl transferase reacts with Dol-P-P:polyP phosphotranferase. The resulting Dol-P passes through the membrane to its cytoplasmic side where it can interact with a new molecule of GDP-mannose. PolyPs re-enter the cell wall together with the newly synthesized mannoproteins via the vesicular transport system where they create complexes with the inactive form of glucan transferase Bg12p (Kalebina et al. 2008). The active complex of Bg12p with polyP exhibits glucan transferase activity and incorporates mannoproteins containing oligoglucan fragments into the cell wall. Thereafter, the exopolyphosphatase of cell wall (Andreeva and Okorokov 1993) or acid phosphatase of this compartment (Kalebina et al. 2008) degrades polyP to P_i and Bgl2p, thence to become the silent form.

Conclusion

The role of polyP in the stress response in yeast is in no doubt, although the mechanism of its participation is still far from fully understood.

The evidence obtained comprises the following:

- PolyP is a storage vehicle for phosphates, which are used by yeast cells under phosphate limitation and which are accumulated under phosphate excess
- PolyP is accumulated when the cell growth is suppressed either by nutritional stresses or stresses caused by toxic heavy metals
- PolyP is a chaperone and is involved in the regulation of enzyme activity through a variety of mechanisms

We assume that polyP is a shunting compound that stores excess energy in the form of phosphoester bonds when the ATP consumption for growth and budding is decreased as a result of growth suppression. It is probable that polyP participates in adaptation to stresses as complexing agents for differently charged compounds.

Acknowledgments The research was supported by Russian Foundation for Basic Research (grant 16-04-00396).

References

Andreeva NA, Kulakovskaya TV, Kulakovskaya EV et al (2008) Polyphosphates and exopolyphosphatases in cytosol and mitochondria of *Saccharomyces cerevisiae* during growth on glucose or ethanol under phosphate surplus. Biochemistry (Mosc) 73:65–69

Andreeva NA, Okorokov LA (1993) Purification and characterization of highly active and stable polyphosphatase from *Saccharomyces cerevisiae* cell envelope. Yeast 9:127–139

Andreeva N, Ryazanova L, Dmitriev V et al (2013) Adaptation of Saccharomyces cerevisiae to toxic manganese concentration triggers changes in inorganic polyphosphates. FEMS Yeast Res 13:463–470

Andreeva N, Ryazanova L, Dmitriev V et al (2014) Cytoplasmic inorganic polyphosphate participates in the heavy metal tolerance of Cryptococcus humicola. Folia Microbiol 59:381–389

Andreeva N, Trilisenko L, Eldarov M et al (2015) Polyphosphatase PPN1 of *Saccharomyces cerevisiae*: switching of exopolyphosphatase and endopolyphosphatase activities. PLoS One 10(3):e0119594. doi:10.1371/journal.pone.0119594

App H, Holzer H (1985) Control of yeast neutral trehalase by distinct polyphosphates and ribonucleic acid. Z Lebensm Unters Forsch 181:276–282

Breus NA, Ryazanova LP, Dmitriev VV et al (2012) Accumulation of phosphate and polyphosphate by *Cryptococcus humicola* and *Saccharomyces cerevisiae* in the absence of nitrogen. FEMS Yeast Res 12:617–624

Breus NA, Ryazanova LP, Suzina NE et al (2011) Accumulation of inorganic polyphosphates in *Saccharomyces cerevisiae* under nitrogen deprivation: stimulation by magnesium ions and peculiarities of localization. Microbiology 80:624–630

De Venditis E, Zahn R, Fasano O (1986) Regeneration of GTP-bound from GDP-bound form of human and yeast ras proteins by nucleotide exchange, stimulatory effect of organic and inorganic polyphosphates. Eur J Biochem 161:473–478

Dmitriev VV, Crowley D, Rogachevsky VV et al (2011) Microorganisms form exocellular structures, trophosomes, to facilitate biodegradation of oil in aqueous media. FEMS Microbiol Lett 315:134–140

Dmitriev V, Tsiomenko A, Kulaev I et al (1980) A cyto-biochemical study of the 'canal' formation the yeast cell wall. Eur J Appl Microbiol Biotechnol 9:211–216

Gray MJ, Wholey W-Y, Wagner NO et al (2014) Polyphosphate is a primordial chaperone. Mol Cell 53:689–699

Hofeler H, Jensen D, Pike MM et al (1987) Sodium transport and phosphorus metabolism in sodium-loaded yeast: simultaneous observation with sodium-23 and phosphorus-31 NMR spectroscopy *in vivo*. Biochemistry 26:4953–4962

Hothorn M, Neumann H, Lenherr ED et al (2009) Catalytic core of a membrane-associated eucaryotic polyphosphate polymerase. Science 324:513–516

Kalebina TS, Egorov SN, Arbatskii NP et al (2008) The role of high-molecular-weight polyphosphates in activation of glucan transferase Bgl2p from *Saccharomyces cerevisiae* cell wall. Dokl Biochem Biophys 420:142–145

Keasling JD, Bertsh L, Kornberg A (1993) Guanosine pentaphosphate phosphohydrolase of *Escherichia coli* is a long-chain exopolyphosphatase. Proc Natl Acad Sci U S A 90: 7029–7033

Kornberg A (1995) Inorganic polyphosphate: toward making a forgotten polymer unforgottable. J Bacteriol 177:491–496

Kulaev IS (1979) The biochemistry of inorganic polyphosphates. Wiley, Chichester

Kulaev IS, Vagabov VM, Kulakovskaya TV (2004) The biochemistry of inorganic polyphosphates. Wiley, Chichester

Kulakovskaya TV, Andreeva NA, Trilisenko LV et al (2004) Two exopolyphosphatases in *Saccharomyces cerevisiae* cytosol at different culture conditions. Proc Biochem 39: 1625–1630

Kulakovskaya TV, Andreeva NA, Trilisenko LV et al (2005) Accumulation of polyphosphates and expression of high molecular weight exopolyphosphatase in the yeast *Saccharomyces cerevisiae*. Biochemistry (Mosc) 70:980–985

Kuroda A (2006) A polyphosphate-Lon protease complex in the adaptation of *Escherichia coli* to amino acid starvation. Biosci Biotechnol Biochem 70:325–331
Lichko LP, Kulakovskaya TV, Kulakovskaya EV et al (2008) Inactivation of PPX1 and PPN1 genes encoding exopolyphosphatases of *Saccharomyces cerevisiae* does not prevent utilization of polyphosphates as phosphate reserve. Biochemistry (Mosc) 73:985–999
Lichko L, Kulakovskaya T, Pestov N et al (2006) Inorganic polyphosphates and exopolyphosphatases in cell compartments of the yeast *Saccharomyces cerevisiae* under inactivation of PPX1 and PPN1 genes. Biosci Rep 26:45–54
Liss E, Langen P (1962) Versuche zur polyphosphat-uberkompensation in heffenzellen nach phosphatverarmung. Arch Microbiol 41:383–392
Lorenz B, Marme S, Müller WEG et al (1994) Preparation and use of polyphosphate-modified zirconia for purification of nucleic acids and proteins. Anal Biochem 216:118–126
Magnusson LU, Farewell A, Nyström T (2005) ppGpp: a global regulator in *Escherichia coli*. Trends Microbiol 13:236–242
Persson BL, Lagerstedt JO, Pratt JR et al (2003) Regulation of phosphate acquisition in *Saccharomyces cerevisiae*. Curr Genet 43:225–244
Pestov NA, Kulakovskaya TV, Kulaev IS (2004) Inorganic polyphosphate in mitochondria of *Saccharomyces cerevisiae* at phosphate limitation and phosphate excess. FEMS Yeast Res 4:643–648
Rao NN, Gómez-García MR, Kornberg A (2009) Inorganic polyphosphate: essential for growth and survival. Ann Rev Biochem 78:605–647
Reddi AR, Jensen LT, Culotta VC (2009) Manganese homeostasis in *Saccharomyces cerevisiae*. Chem Rev 109:4722–4732
Reusch RN (1992) Biological complexes of poly-β-hydroxybutyrate. FEMS Rev 103:119–130
Ryazanova L, Zvonarev A, Rusakova T et al (2015) Manganese tolerance in yeasts involves polyphosphate, magnesium, and vacuolar alterations. Folia Microbiol. doi:10.1007/s12223-015-0440-9
Schröder HC, Lorenz B, Kurz L et al (1999) Inorganic polyphosphate in eukaryotes: enzymes, metabolism and function. Prog Mol Subcell Biol 23:45–81
Shabalin YA, Kulaev IS (1989) Solubilization and properties of yeast dolichylpyrophosphate: polyphosphate phosphotransferase. Biokhimia (Mosc) 54:68–75
Shabalin YA, Naumov AV, Vagabov VM et al (1984) The discovery of new enzyme activity dolychildiphosphate polyphosphate phosphotransferase in yeast. Dokl Acad Nauk SSSR 278:482–485
Sharma UK, Chatterji D (2010) Transcriptional switching in Escherichia coli during stress and starvation by modulation of sigma activity. FEMS Microbiol Rev 34:646–657
Tomaschevsky AA, Ryasanova LP, Kulakovskaya TV et al (2010) Inorganic polyphosphate in the yeast *Saccharomyces cerevisiae* with a mutation disturbing the function of vacuolar ATPase. Biochemistry (Mosc) 75:1052–1054
Trilisenko LV, Vagabov VM, Kulaev IS (2002) The content and chain length of polyphosphate from vacuoles of *Saccharomyces cerevisiae* VKM Y-1173. Biochemistry (Mosc) 67:592–596
Vagabov VM, Trilisenko LV, Kulakovskaya TV et al (2008) Effect of carbon source on polyphosphate accumulation in Saccharomyces cerevisiae. FEMS Yeast Res 8:877–882
Vagabov VM, Trilisenko LV, Shchipanova IN et al (1998) Changes in inorganic polyphosphate length during the growth of *Saccharomyces cerevisiae*. Microbiologiia (In Russian) 67:153–157
Vagabov VM, Trilisenko LV, Kulaev IS (2000) Dependence of inorganic polyphosphate chain length on the orthophosphate content in the culture medium of the yeast *Saccharomyces cerevisiae*. Biochemistry (Mosc) 65:349–355
Wolska-Mitaszko B (1997) Trehalases from spores and vegetative cells of yeast *Saccharomyces cerevisiae*. J Basic Microbiol 37:295–303

Yeast Polyphosphatases PPX1 and PPN1: Properties, Functions, and Localization

2

Nadeshda Andreeva, Lidia Lichko, Ludmila Trilisenko, Ivan V. Kulakovskiy, and Tatiana Kulakovskaya

Abstract

The *PPX1* and *PPN1* genes of *Saccharomyces cerevisiae* encode the enzymes that hydrolyze inorganic polyphosphates (polyP) of different chain lengths including tripolyphosphate. They are divalent metal ion dependent. PPX1 is an exopolyphosphatase splitting P_i from polyP chain end. PPN1 displays exopolyphosphatase and endopolyphosphatase activities in the presence of cobalt and magnesium ions, respectively. PPN1 prefers long-chain polyP, while PPX1 prefers short-chain polyP. Commonly, PPX1 is localized in the cytoplasm and mitochondrial matrix, while PPN1 is localized in the vacuoles, nuclei, and mitochondrial membrane. PPN1 appears in the cytoplasm at the early growth stage under phosphate excess. The *PPX1* or *PPN1* knockouts increase polyP content in *S. cerevisiae*. Δ*PPN1* mutants contain polyP with longer chains, while Δ*PPX1* does not demonstrate polyP chain elongation. PPX1 overexpression has no effect on polyP content and chain length. In turn, *PPN1* overexpression decreases polyP content. Both PPX1 and PPN1 catalyze the same reaction, but there is no similarity between the amino acid sequences of these enzymes.

N. Andreeva • L. Lichko • L. Trilisenko
Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia

I.V. Kulakovskiy
Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilova 32, Moscow 119991, Russia

Vavilov Institute of General Genetics, Russian Academy of Sciences, Gubkina, 3, Moscow 119991, Russia

T. Kulakovskaya (✉)
Laboratory of Regulation of Biochemical Processes, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia
e-mail: alla@ibpm.pushchino.ru

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_2

Furthermore, the human ortholog of PPX1, the prune protein, has an exopolyphosphatase activity, but neither functional nor structural homologs of PPN1 have been found in higher eukaryotes.

2.1 Introduction

Inorganic polyphosphate (polyP, $polyP_n$, where n is the average number of phosphate residues in the polymer chain) is a linear polymer containing a few to several hundred orthophosphate residues linked by energy-rich phosphoanhydride bonds. This polymer is an essential component of yeast cells containing polyP in all cellular compartments (Kulaev et al. 2004). Many enzymes are involved in polyP metabolism in the yeast *Saccharomyces cerevisiae*. Exopolyphosphatase (polyphosphate phosphohydrolase EC 3.6.1.11) cleaves P_i from the end of the polyP chain:

$PolyP_n + H_2O \rightarrow PolyP_{n-1} + P_i$

Endopolyphosphatase (polyphosphate depolymerase, EC 3.6.1.10.) splits long polyP molecules into shorter ones:

$PolyP_n + H_2O \rightarrow$ Oligopolyphosphate

The first data on exopolyphosphatase and endopolyphosphatase activities in fungi were reported long ago (Kitasato 1928; Malmgren 1952; Kritsky et al. 1972). Later, a highly active and stable exopolyphosphatase was purified from *Saccharomyces cerevisiae* (Andreeva et al. 1990). The enzyme was inactive against pyrophosphate, *p*-nitrophenylphosphate, and nucleoside triphosphates, suggesting it could be a specific exopolyphosphatase. The first gene encoding the exopolyphosphatase in yeasts, PPX1, was cloned and sequenced in 1995 (Wurst et al. 1995). In the knockout mutant, the polyphosphatase activity has not ceased completely (Sethuraman et al. 2001). This activity was provided by PPN1, which was shown to be an endopolyphosphatase (Sethuraman et al. 2001). Furthermore, it also displays an exopolyphosphatase activity (Andreeva et al. 2006).

An exopolyphosphatase activity was observed in the vacuoles of the double Δ*PPX1*Δ*PPN1* mutant (Lichko et al. 2006). However, the respective enzyme has not yet been identified. A previously unknown endopolyphosphatase, probably encoded by the *DDP1* gene (Lonetti et al. 2011), was purified from the same double mutant (Lichko et al. 2010).

The amino acid sequences of PPN1 and PPX1 are very dissimilar. The enzymes have different cell localization and substrate specificity suggesting different functions in yeast cells. This chapter comparatively describes the localization, properties, and functions of these enzymes in *S. cerevisiae*.

2.2 Structural Features

The PPX1_yeast gene is located in ChrVIII. PPX1 (UniProt P38698/PPX1_YEAST) is a single-subunit protein of 397 amino acid residues with the calculated molecular mass of 45.05 kD. Its molecular mass is in the range of 40–45 kD according to electrophoresis and gel filtration data; the differences are attributed to the technical limitations of experimental procedures. The *PPX1* gene has no introns; posttranslational

modification of the respective protein has not been reported. PPX1 belongs to the DHH family of phosphoesterases including: the family-2 inorganic pyrophosphatases (PPases) found in Gram-positive bacteria; prune proteins of insects and mammals; and RecJ, a single-stranded DNA exonuclease (Ugochukwu et al. 2007). The high-resolution 3D structure of the yeast PPX1 was obtained by X-ray crystallography. The active-site region was found to be highly similar to that of the family-2 inorganic pyrophosphatases. PPX1 has two structural domains. A large extended channel formed at the interface between the N- and C-terminal domains is lined with positively charged amino acids and serves as a conduit for polyP and a phosphate hydrolysis site (Ugochukwu et al. 2007). These features provide a possible explanation for the processive mechanism of polyP hydrolysis and negligible pyrophosphatase activity of PPX1 (Ugochukwu et al. 2007). Mutations in Asp127, His148, His149 (conserved between PPX1 and PPase), and Asn35 (His in PPase) (Tammenkoski et al. 2008) notably decrease the exopolyphosphatase activity of PPX1.

PPN1_yeast gene is located in ChrIV. The protein PPN1 (UniProt Q04119/PPN1_YEAST) is a homotetramer. The *PPN1* gene encodes a polypeptide with the predicted molecular mass of 78.344 kD, whereas the molecular mass of the mature monomer is about 33–35 kD (Sethuraman et al. 2001; Andreeva et al. 2006). The specific proteolysis by vacuolar protease is needed for enzyme maturation and activity (Sethuraman et al. 2001). PPN1 contains several putative sites of glycosylation and ubiquitination. N-glycosylation is essential for protease-mediated maturation (Shi and Kornberg 2005). When purified, PPN1 often forms huge aggregates with a molecular mass of 800 kD and more (Andreeva et al. 2004). The presence of polyP is necessary for enzyme stabilization and activity of the homotetramer form (Andreeva et al. 2004). There is no direct experimental data on PPN1 3D structure.

2.3 Substrate Specificity, Kinetics, Inhibitors and Activators

The PPX1 and PPN1 of *S. cerevisiae* hydrolyze polyP of different chain lengths with the release of P_i but fail to hydrolyze *p*-nitrophenylphosphate (the substrate of phosphatases with a broad spectrum of action), ATP and other nucleoside triphosphates, and PP_i (Andreeva and Okorokov 1993; Andreeva et al. 1996, 2004). The optimal pH of the PPX1 and PPN1 enzymes is nearly 7.0 (Andreeva et al. 1996, 2004).

The reported specific activities of the purified enzymes varied depending on purification methods and assay conditions. Since it is difficult to estimate precisely the molecular mass of polyP substrate, a special unit is used to unify the polyphosphatase activity measures. A unit (U) is the amount of the enzyme forming 1 μmole of P_i per 1 min. The exopolyphosphatase activities with $polyP_{208}$ (polyP with an average chain length of 208 phosphate residues) of 300 U/mg protein and 900 U/mg protein were reported for PPX1 (Andreeva et al. 1996) and PPN1 (Andreeva et al. 2015a), respectively.

PPN1 is more effective at hydrolyzing long-chain polyP, and PPX1 is more effective at hydrolyzing short-chain polyP (Table 2.1). The enzyme-substrate affinities are considerably higher for longer-chain polyP (Table 2.1). For PPX1, the dependence of substrate hydrolysis rate from substrate concentration approximates to the Michaelis-Menten equation (at a constant concentration of magnesium ions)

Table 2.1 Substrate specificity and apparent Km values of PPX1 (Andreeva and Okorokov 1993; Andreeva et al. 1996) and PPN1 (Andreeva et al. 2004, 2015a, b)

	PPX1[a]		PPN1[b]	
Substrate	Specific activity, %	Km, μM	Specific activity, %	Km, μM
$PolyP_3$	160	170–300	14	1,100
$PolyP_9$	113	15–19	45	
$PolyP_{15}$	118	11	88	75
$PolyP_{25}$	105		100	
$PolyP_{45}$	109		100	
$PolyP_{208}$	100	0.9–1.2	100	3.5

[a]In the presence of 2.5 mM Mg^{2+}

[b]In the presence of 0.1 mM Co^{2+}

(Andreeva et al. 1996). The kinetics of PPN1 does not follow the Michaelis-Menten equation (Andreeva et al. 2004). Table 2.1 presents the concentrations of substrates providing the half-maximal reaction velocity.

The PPX1 and PPN1 enzymes are divalent cation dependent. Magnesium, manganese, and cobalt ions stimulate the activity of both enzymes, while calcium, iron, cuprum have no stimulatory effect (Andreeva and Okorokov 1993; Andreeva et al. 1996, 2004). Zinc ions stimulate the activity at 0.05–0.1 mM but have an inhibitory effect at higher concentrations (Andreeva et al. 1996, 2004). The concentration dependences of exopolyphosphatase activity on divalent cations are different for PPX1 and PPN1 (Fig. 2.1). Monovalent cations stimulate both enzymes; NH_4 is most effective (Table 2.2).

The initial kinetic models for PPX1 assumed that tripolyphosphate can be hydrolyzed both by 1:1 and 1:2 $PolyP_3/Mg^{2+}$ complexes; however, the optimal substrate is the 1:1 complex, while the higher concentrations of magnesium ions decrease the hydrolysis rate (Kulakovskaya et al. 1999). A single tight binding site for Mg^{2+} (*Kd* of 24 μM) was detected in PPX1 by equilibrium dialysis (Tammenkoski et al. 2007). The steady-state kinetic analysis of tripolyphosphate hydrolysis revealed a second site that binds Mg^{2+} in the millimolar range and modulates substrate binding (Tammenkoski et al. 2007).

The PPX1 and PPN1 have similar responses to the selected inhibitors. They are insensitive to molybdate (the common phosphohydrolase inhibitor) and fluoride (the inhibitor of pyrophosphatases). They are not inhibited by many known ATPase inhibitors including azide, oligomycin, orthovanadate, N,N′-dicyclohexylcarbodiimide, diethylstilbestrol, and nitrate. SH reagents such as N-ethylmaleimide and iodoacetamide have little or no effect on exopolyphosphatase activity. Insensitivity to orthovanadate suggests the inability of exopolyphosphatases to form a phosphorylated intermediate during the reaction of polyP hydrolysis. Heparin suppresses the activities of both PPX1 and PPN1 (see Table 2.2 for complete data and references).

PPX1 cleaves the terminal phosphate from adenosine tetraphosphate and guanosine tetraphosphate (Kulakovskaya et al. 1997; Guranowski et al. 1998). The

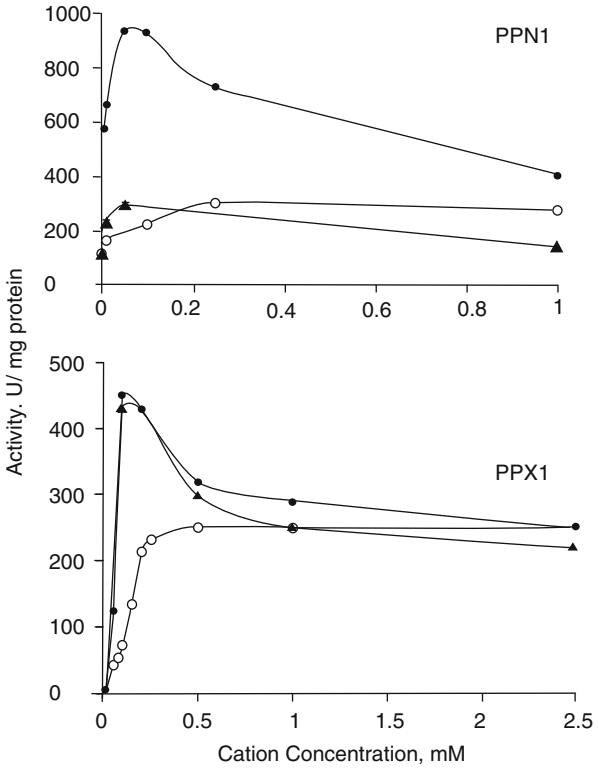

Fig. 2.1 The effects of Co^{2+} (*black circles*), Mn^{2+} (*black triangles*), and Mg^{2+} (*white circles*) on the exopolyphosphatase activities of PPN1 with $polyP_{208}$ (Andreeva et al. 2015b) and PPX1 with $polyP_{15}$ (Andreeva et al. 1996)

Table 2.2 The effects of monovalent cations and heparin on the exopolyphosphatase activities of purified PPX1 (Andreeva et al. 1996) and PPN1 (Andreeva et al. 2004, 2015a, b) with $PolyP_{208}$ (2.5 mM PolyP as phosphorus)

Effectors	PPX1 specific activity, % of the control	PPN1 specific activity, % of the control
Control	100	100
NaCl, 200 mM	120	140
KCl, 200 mM	120	140
NH_4Cl, 200 mM	140	170
Heparin, 0.001 mg/ml	5	35
Heparin, 0.01 mg/ml	0	5

The reaction mixture contained 2.5 mM Mg SO_4 and 0.1 mM $CoSO_4$ in case of PPX1 and PPN1, respectively

activity with adenosine-5′-tetraphosphate and guanosine-5′-tetraphosphate is twice higher than with $PolyP_{15}$; the apparent K_m values are 80–100 μM (Kulakovskaya et al. 1997).

The PPX1 enzyme does not demonstrate an endopolyphosphatase activity in the presence of magnesium or cobalt ions. There is no chain length shift during polyP

hydrolysis by PPX1 (Fig. 2.2) which agrees with the originally suggested processive mechanism of polyP hydrolysis (Wurst and Kornberg 1994).

The PPN1 enzyme displays both endo- and exopolyphosphatase activities (Fig. 2.2, Table 2.1). The endopolyphosphatase and exopolyphosphatase activities are low in the absence of divalent cations (Andreeva et al. 2015a). The exopolyphosphatase activity of PPN1 is predominant in the presence of Co^{2+}, while the endopolyphosphatase activity is predominant in the presence of Mg^{2+} (Andreeva et al. 2015a). In the presence of Co^{2+}, the enzyme effectively releases P_i (Fig. 2.1). Simultaneously, the chain length of polyP decreases from ~208 to ~15 phosphate residues (Fig. 2.2). P_i release is insignificant in the presence of Mg^{2+} (Fig. 2.1), though the substrate chain length decreases from ~208 to ~45–60 phosphate residues in the first 30 min of the reaction and then remains at a fixed level (Fig. 2.2). Probably, $polyP_{60}$ is not a suitable substrate for PPN1 in the presence of Mg^{2+}. ATP had no influence on exopolyphosphatase activity irrespective of the cation but inhibited endopolyphosphatase activity in the presence of Mg^{2+} but not Co^{2+}. ADP inhibited both reactions in the presence of Co^{2+} but activated them in the presence of Mg^{2+} (Andreeva et al. 2015a).

The PPX1 and PPN1 enzymes are different in molecular mass, substrate specificity, and dependency on bivalent cations. These differences are convenient criteria for detecting these enzymes in subcellular fractions and assessing the changes in their activities under varied cultivation conditions (Andreeva et al. 2001; Kulakovskaya et al. 2004). The differences in physicochemical properties suggest the specific roles of PPX1 and PPN1 in yeast phosphorus metabolism.

2.4 Cellular Localization

The specific exopolyphosphatases have been found in the cellular compartments in *S. cerevisiae* (Lichko et al. 2003, 2006). Exopolyphosphatases from the cell envelope (Andreeva et al. 1990; Andreeva and Okorokov 1993), cytosol (Andreeva et al. 1996, 2004), vacuolar sap (Andreeva et al. 1998), mitochondrial matrix (Lichko et al. 2000), and nuclei (Lichko et al. 2004a) have been purified and characterized. The comparative analysis of physicochemical properties of exopolyphosphatases of the cell envelope, cytoplasm, and mitochondrial matrix revealed their strong similarity to the properties of the exopolyphosphatase from the *S. cerevisiae* homogenate (Wurst and Kornberg 1994) encoded by the PPX1 gene (Wurst et al. 1995). It suggests that the same PPX1 enzyme is responsible for exopolyphosphatase activities in the above cell compartments.

The endopolyphosphatase PPN1 was initially purified from the homogenate of the Δ*PPX1* mutant (Sethuraman et al. 2001). Later, the high-molecular exopolyphosphatase of the cytoplasm was purified from the wild strain (Andreeva et al. 2004). The mass spectrum of the tryptic hydrolysate showed that the corresponding enzyme was also a product of the *PPN1* gene (Andreeva et al. 2006). The properties of the exopolyphosphatase from vacuolar sap (Andreeva et al. 1998) were highly similar to those of the high-molecular exopolyphosphatase from the

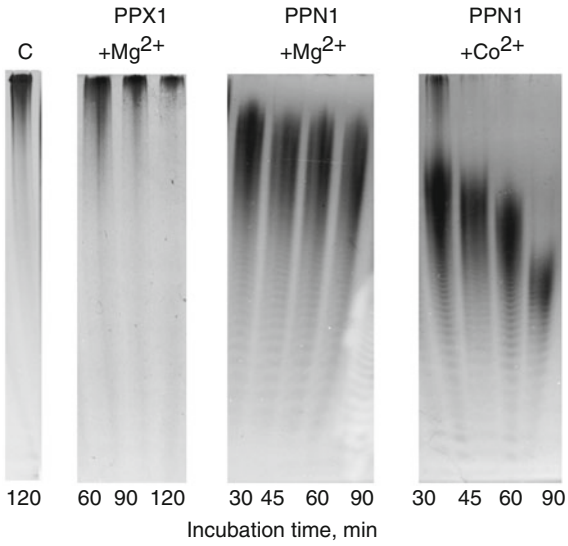

Fig. 2.2 The chain length of $polyP_{208}$ during incubation with PPX1 (in the presence of 2.5 mM Mg^{2+}) or PPN1 (in the presence of 0.25 mM Mg^{2+} or 0.1 mM Co^{2+}). *C* control, the substrate $polyP_{208}$ was incubated for 120 min without enzyme. PolyP PAGE was performed in 24% polyacrylamide gel with 7M urea; toluidine blue staining

Table 2.3 Exopolyphosphatase activities (mU/mg protein) in the subcellular fractions of parent strain and knockout mutants of *Saccharomyces cerevisiae* (kindly provided by A. Kornberg) at the stationary growth stage in the YPD medium (Lichko et al. 2006)

	Subcellular fraction				
Yeast strain	Cytoplasm	Nuclei	Vacuoles	Mitochondria, soluble fraction	Mitochondria, membrane fraction
Parent strain	130	100	375	135	100
Δ*PPX1*	80	80	370	35	80
Δ*PPN1*	45	15	20	15	0
Δ*PPX1*/Δ*PPN1*	0	0	55	0	0

cytoplasm. This suggested that the vacuolar enzyme was also a product of the *PPN1* gene.

The exopolyphosphatase activities in subcellular fractions of the Δ*PPX1* and Δ*PPN1* mutants were studied to assess the localization of PPX1 and PPN1 in the cells (Table 2.3, Fig. 2.3). In the Δ*PPX1* strain, exopolyphosphatase activities in the nuclei, vacuoles, and mitochondrial membranes did not change compared to the parent strain (Lichko et al. 2006). The exopolyphosphatase activity was negligible in the Δ*PPX1* cell envelope extract; PPX1 was absent in the cytoplasm and mitochondrial matrix (Lichko et al. 2004b, 2006; Pestov et al. 2005). Instead, these compartments contained a high-molecular exopolyphosphatase with the properties similar to those of the *PPN1* gene product (Lichko et al. 2004a, b; Pestov et al. 2005). The exopolyphosphatase activity was absent in the mitochondrial membranes of the Δ*PPN1* mutant and drastically decreased in vacuoles and nuclei

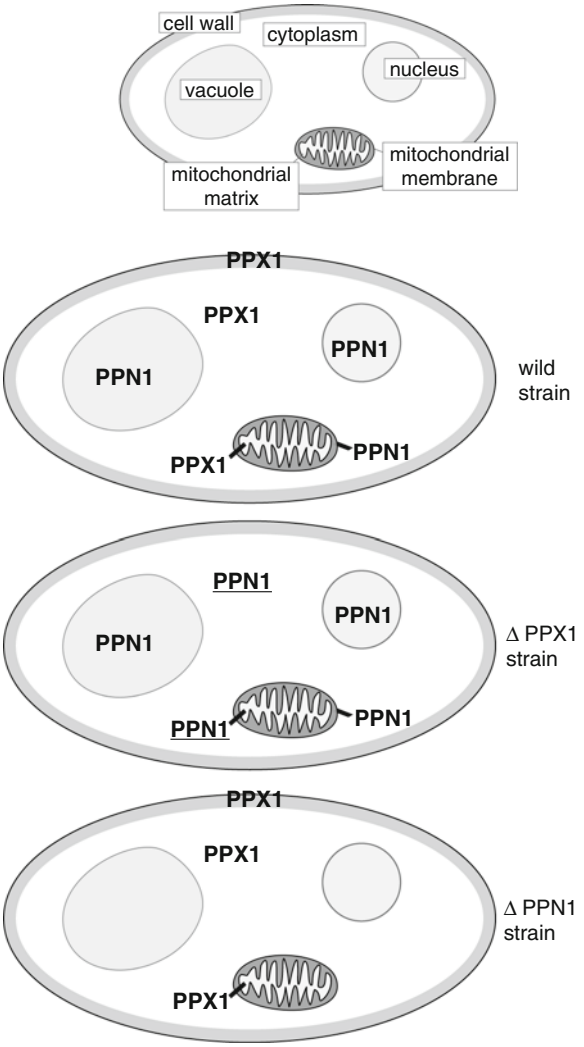

Fig. 2.3 The localization of PPX1 and PPN1 in the cells of wild strain and knockout mutants

(Pestov et al. 2005; Lichko et al. 2006). These data demonstrate that PPN1 is normally localized in vacuoles, nuclei, and mitochondrial membrane, whereas PPX1 is localized in the cell envelope, cytoplasm, and mitochondrial matrix (Fig. 2.3). Note that PPN1 is localized primarily in vacuoles but is also responsible for the exopolyphosphatase activity of nuclei and mitochondrial membranes.

The exopolyphosphatase activities of nuclei and mitochondria have specific features regarding the kinetics and dependence on divalent metal cations (Lichko et al. 2006). Probably, these features may be attributed to the posttranslational modifications of proteins or, in case of mitochondria, to the effect of membrane association (Lichko et al. 1998).

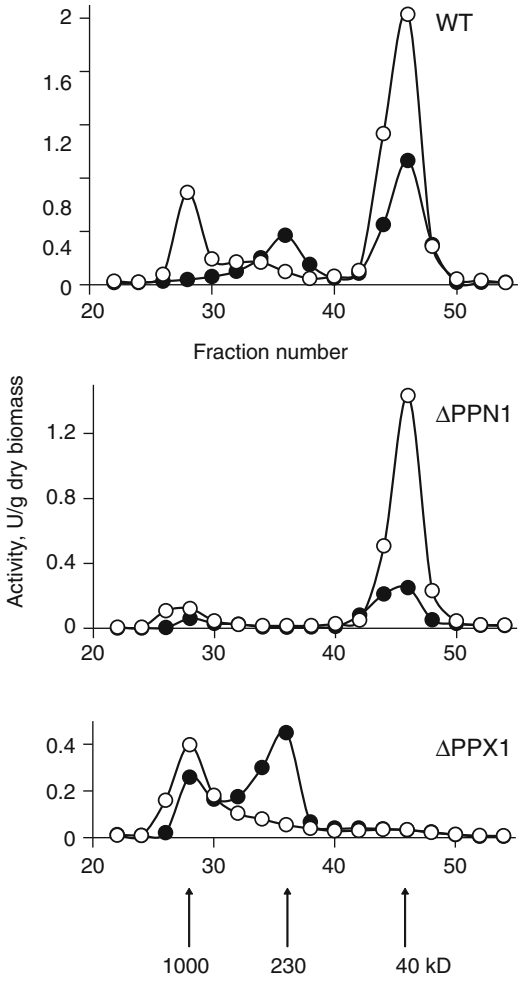

Fig. 2.4 Exopolyphosphatase activities in Sephacryl S-300 gel filtration fractions of cytosol preparations from wt, Δ*PPN1*, and Δ*PPX1* strains of *S. cerevisiae*. Exopolyphosphatase activities were measured with 2 mM $polyP_{208}$ and 0.1 mM Co^{2+}. *White circles* logarithmic growth stage, *black circles* stationary growth stage (Lichko et al. 2004b)

2.5 Interplay of PPX1 and PPN1

The levels of PPX1 and PPN1 expression are mutually dependent. In the *PPX1* knockout mutant, PPN1 appears in the cytoplasm (Fig. 2.4) and mitochondrial matrix (Lichko et al. 2004a, b; Pestov et al. 2005). A strikingly different effect is observed in the Δ*PPN1* mutant, where the activity of PPX1 in the cytoplasm drastically decreases in the stationary growth phase (Fig. 2.4).

In cytoplasm, the activity of PPN1 increases and the activity of PPX1 decreases in the wild strain at the early growth stage under phosphate excess (Table 2.4). The activity of PPN1 in the cytoplasm under phosphate deficiency is low, irrespective of the growth stage (Table 2.4). A notable increase in PPN1 exopolyphosphatase activity in the cytoplasm was observed in the media with both glucose and ethanol (Kulakovskaya et al. 2004; Andreeva et al. 2008) under phosphate surplus (when

Table 2.4 The effects of growth stage and P_i concentration on the activity of PPX1 and PPN1 in the preparation of the cytoplasm of *S. cerevisiae* wild strain VKM Y-1173 (Kulakovskaya et al. 2004)

Growth stage	Cultivation conditions and P_i concentration in the medium	PPX1, U/g of dry biomass	PPN1 U/g dry biomass
Stationary stage	20 mM P_i	7.0	0.5
Stationary stage	1 mM P_i	16.0	1.0
Early logarithmic stage, 4 h of growth	Re-inoculation from 1 mM P_i to 20 mM P_i	10.4	9.0
Early logarithmic stage, 4 h of growth	Re-inoculation from 20 mM P_i to 20 mM P_i	8.5	10.4
Early logarithmic stage, 4 h of growth	Re-inoculation from 1 mM P_i to 1 mM P_i	15.0	2.7

yeast cells are re-inoculated from phosphate-deficient to complete medium). Under phosphate surplus in the ethanol-containing medium, PPN1 also appears in the soluble mitochondrial fraction, in contrast to the cultivation in the glucose-containing medium, where this fraction contains PPX1 but not PPN1 (Andreeva et al. 2008). The expression and localization of PPN1 seem to be under the control of phosphate concentration, carbon source, and growth stage.

The above facts suggest interdependence between the expression and localization of polyphosphatases in yeasts, but the details of this mechanism are still obscure. Probably, the specific signal compounds such as adenosine tetraphosphate and inositol polyphosphates are involved in this process.

2.6 Cellular PolyP Under PPX1 and PPN1 Knockout

The polyP content in the cells of Δ*PPX1* and Δ*PPN1* mutants is anticorrelated with the total exopolyphosphatase activity (Fig. 2.5). The double Δ*PPX1*Δ*PPN1* mutant demonstrates the most noticeable increase of polyP content in cells, mostly in the acid-soluble polyP fraction.

The Δ*PPX1* knockout mutant does not exhibit any notable changes in polyP chain length of subcellular fractions (Fig. 2.6). At the same time, the Δ*PPN1* mutant contains longer-chain PolyP in the cytoplasm, mitochondria, and vacuoles compared to the parent strain (Fig. 2.6). Furthermore, the Δ*PPN1* mutant demonstrates the absence of membrane-bound exopolyphosphatase, the accumulation of long-chain polyP in mitochondria, and the defects in mitochondrial respiration, lactate, and ethanol consumption (Pestov et al. 2005).

The level of PPX1 activity in the cytoplasm of the wild strain increases twofold at the stationary growth stage in a P_i-deficient medium, but the level of PPN1 activity does not change (Kulakovskaya et al. 2004) (Table 2.4). However, the Δ*PPX1*, Δ*PPN1*, and Δ*PPX1*Δ*PPN1* mutants have no growth defects in a P_i-deficient medium and maintain relatively constant P_i levels in the cytoplasm (Lichko et al. 2008). It suggests that PPX1 and PPN1 are able to functionally replace each other in

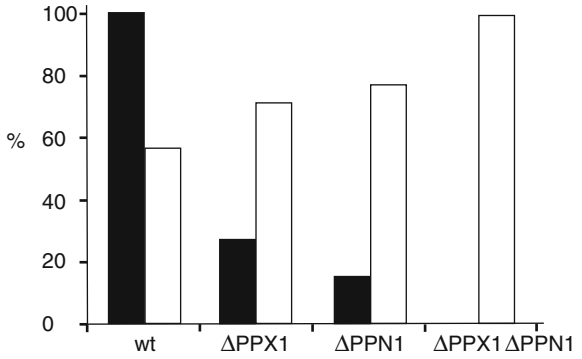

Fig. 2.5 Exopolyphosphatase activity (*dark bars*) and the total content of polyP (*white bars*) in the stationary growth stage in the cells of wt and knockout mutants Δ*PPX1*, Δ*PPN*, and Δ*PPN1*Δ*PPX1* (Kulakovskaya et al. 2006)

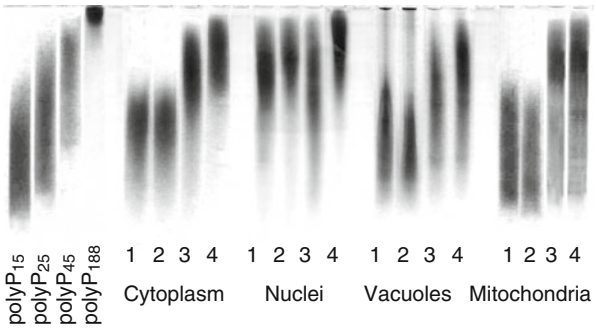

Fig. 2.6 The chain length of polyP from purified fraction of cytoplasm, vacuoles, nuclei, and mitochondria. PolyP PAGE was performed in 24 % polyacrylamide gel with 7 M urea; toluidine blue staining. PolyP markers: commercial polyP with the average chain lengths of 15, 25, 45 phosphate residues from Sigma (USA) and PolyP with an average chain length of 188 phosphate residues from Monsanto (USA). The strains used: (*1*) wt, (*2*) Δ*PPX1*, (*3*) Δ*PPN1*, (*4*) Δ*PPX1*Δ*PPN1* (Lichko et al. 2006)

polyP consumption. This idea is confirmed by the data on the changes in the average polyP chain length of the mutants under P_i-deficiency. In the cytoplasm of the Δ*PPN1* mutant, the average chain length of polyP was not changed at a decrease in polyP content. This agrees with the processive mechanism of PPX1, which consumes polyP in the Δ*PPN1* mutant. On the contrary, in the Δ*PPX1* mutant under P_i-deficiency, the long-chain polyPs are depleted first and the short-chain polyPs are hydrolyzed later (Lichko et al. 2008). This agrees with the PPN1 substrate specificity.

Interestingly, the high activities of both PPX1 and PPN1 during cultivation of the wild strain of *S. cerevisiae* under P_i excess cannot prevent polyP accumulation. Probably, the compartmentalization and association of polyP with cations and other compounds are responsible for the stability of polyP levels in yeast cells.

Table 2.5 Exopolyphosphatase activities and polyP content in PPX1 and PPN1 overproducing strains of *Saccharomyces cerevisiae* (Eldarov et al. 2013; Lichko et al. 2014)

Strain	Exopolyphosphatase in cellular extract, U/mg protein	Acid-soluble PolyP, μmol P/g of wet biomass	Acid-insoluble PolyP, μmol P/g of wet biomass
CRN (Δ*PPN1* strain)	0.06	54	110
CRN/pMB1_PPN1 Sc (PPN1 overproducing strain)	1.7	6.2	42
CRN/pMB1_PPX1 Sc (PPX1 overproducing strain)	2.0	73	73

2.7 Cellular PolyP Under PPX1 and PPN1 Overexpression

The *PPN1* strain was transformed by the vector containing the *PPX1* or *PPN1* genes under a strong constitutive promoter of glycerol aldehyde triphosphate dehydrogenase of *S. cerevisiae* (Andreeva et al. 2015b; Lichko et al. 2014). The exopolyphosphatase activities in the transformants increased drastically (Table 2.5). The overexpressing strains had no growth defects under phosphate deficiency or phosphate excess.

The recombinant PPN1 appears in the mature form and is localized mainly in the cytoplasm (40 %) and vacuoles (20 %) (Andreeva et al. 2015b), which resembles the wild strain under phosphate excess, where PPN1 level also increases (see Sect. 2.4). The recombinant PPX1 is localized mainly in the cytoplasm as the native PPX1 (Lichko et al. 2014).

The polyP content decreases in case of PPN1 overexpression and changes insignificantly in case of PPX1 overexpression (Table 2.5), suggesting different roles of PPN1 and PPX1 in the polyP metabolism of *S. cerevisiae*. PPX1 seems to be involved in utilization of polyP as a phosphate reserve under P_i limitation. The putative primary role of PPN1 is to split the long-chain polyP into shorter chains. It is especially important at early growth stages, when polyP might be an additional energy source for rapid growth (Trilisenko and Kulakovskaya 2014). Probably, the energy of the phosphoester bond of short-chain polyP can be used for ATP synthesis or directly for membrane energization.

2.8 Orthologous Proteins in Other Organisms

Bioinformatics databases can be used to assess orthologous proteins for PPN1 and PPX1. In particular, Ensembl portal (Cunningham et al. 2015) provides an overview of orthologous proteins. For PPN1, the only close orthologs are sphingomyelin and

calcineurin-like phosphoesterases of higher eukaryotes. Furthermore, the BLASTp search for the PPN1 sequence reports two conserved domains (Marchler-Bauer et al. 2015) overlapping the putative metal-binding and active sites: MPP_ASMase (acid sphingomyelinase and related proteins) and Metallophos (calcineurin-like phosphoesterase) matching the PPN1 domains with very high identity.

The human acid sphingomyelinase-like phosphodiesterase (EC 3.1.4.12) performs the following reaction:

Sphingomyelin + H(2)O ⇔ N − acylsphingosine + choline phosphate.

The protein is membrane bound and belongs to the nucleotide pyrophosphatase/phosphodiesterase family. This enzyme is potentially significant for the digestive system, and mutations in the coding sequence have been found in colon cancer cells (Duan 2006).

However, there is no direct knowledge of whether these proteins can hydrolyze polyP.

It is worth noting that the closest orthologs of PPN1 in higher eukaryotes have only a 15–20 % identity according to Ensembl. At the same time, the PPN1 identity between different yeast species is also limited (from 30 to 50 %, see Table 2.6).

On the contrary, the closest ortholog of PPX1, the prune protein of higher eukaryotes, has up to 25–30 % identity with PPX1 (Table 2.7). The human prune (h-prune) hydrolyzes short-chain polyP; the best substrates are tripoly- and tetrapolyphosphates and nucleoside 5′-tetraphosphates (Tammenkoski et al. 2008). The activity with polyP of 25 and more phosphate residues is tenfold lower than that with $polyP_3$, whereas pyrophosphate and nucleoside triphosphates are not hydrolyzed. The dramatic difference in substrate specificity between prune and PPX1 may be explained by the peculiarities of active center structure. The reaction requires a divalent metal cofactor such as Mg^{2+}, Co^{2+}, or Mn^{2+}, which activates both the enzyme and the substrate. Notably, the exopolyphosphatase activity of h-prune is suppressed by the nm23-H1 protein, long-chain polyphosphates, and pyrophosphate, which potentially acts as physiological regulators (Tammenkoski et al. 2008).

Specifically, nm23-H1 is a metastasis suppressor (Marino et al. 2011) and, in combination with h-prune and glycogen synthase kinase-3 (Gsk-3β), promotes cell migration (Carotenuto et al. 2014) and probably participates in tumorigenesis and metastasis (Galasso and Zollo 2009).

Interestingly, the high expression of prune, at least at the RNA level, is not a specific characteristic of cancer cells and cell lines. FANTOM5 CAGE data (Forrest et al. 2014) shows the high expression of h-prune in platelets, brain tissues, and smooth muscle samples; the average expression levels in cancer and normal cells are comparable (see Fig. 2.7). Cell lines (mostly cancer derived) demonstrate the expression level comparable to that of normal primary cells. It is interesting that the expression variance is quite notable in all cases, though h-prune expression is generally higher in tissue samples.

Table 2.6 The identity matrix for PPN1 orthologs reviewed in UniProt (based on ClustAl multiple alignment (Goujon et al. 2010)

	PPN1_ SCHPO	PPN1_ CRYNJ	PPN1_ NEUCR	PPN1_ YARLI	PPN1_ DEBHA	PPN1_ KLULA	PPN1_ YEAST	PPN1_ CANGA
PPN1_SCHPO	100	34	33	35	34	34	37	35
PPN1_CRYNJ	34	100	35	33	35	34	33	34
PPN1_NEUCR	33	35	100	38	37	35	37	36
PPN1_YARLI	35	33	38	100	42	45	41	42
PPN1_DEBHA	34	35	37	42	100	49	49	46
PPN1_KLULA	34	34	35	45	49	100	51	52
PPN1_YEAST	37	33	37	41	49	51	100	55
PPN1_CANGA	35	34	36	42	46	52	55	100

Each cell shows the identity percentage between two protein sequences. UniProt protein IDs are shown
PPN1_YEAST – *Saccharomyces cerevisiae*, PPN1_NEUCR – *Neurospora crassa*, PPN1_SCHPO – *Schizosaccharomyces pombe*, PPN1_KLULA – *Kluyveromyces lactis*, PPN1_CRYNJ – *Cryptococcus neoformans*, PPN1_CANGA – *Candida glabrata*, PPN1_DEBHA – *Debaryomyces hansenii*, PPN1_YARLI – *Yarrowia lipolytica*

Table 2.7 The identity matrix for selected PPX1 orthologs listed in Ensembl

	Drosophila melanogaster	*Homo sapiens*	*Danio rerio*	*Cryptococcus neoformans*	*Neurospora crassa*	*Acremonium chrysogenum*	*Candida albicans*	*Saccharomyces cerevisiae*
Drosophila melanogaster	100	32	33	26	25	30	23	26
Homo sapiens	32	100	50	26	26	29	25	25
Danio rerio	33	50	100	27	26	30	27	25
Cryptococcus neoformans	26	26	27	100	29	28	27	25
Neurospora crassa	25	26	26	29	100	52	27	29
Acremonium chrysogenum	30	29	30	28	52	100	31	30
Candida albicans	23	25	27	27	27	31	100	34
Saccharomyces cerevisiae	26	25	25	25	29	30	34	100

The identity percentage values in each cell are based on ClustAl multiple alignment (Goujon et al. 2010)

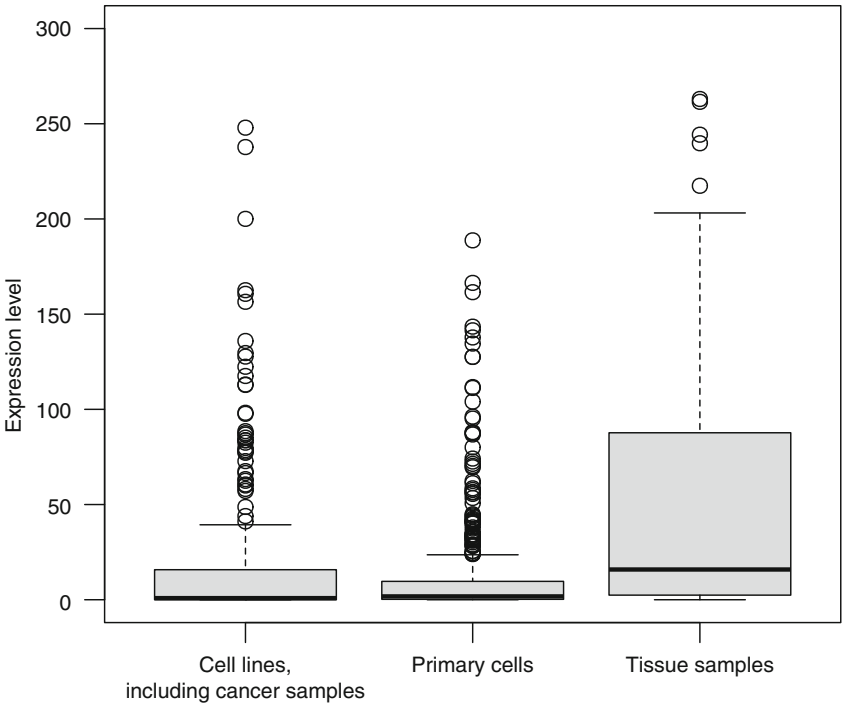

Fig. 2.7 The standard box-and-whiskers plot displaying h-prune expression level in different samples. *Y axis*: RLE-normalized CAGE-tags per million, see Forrest et al. (2014) for details

Conclusion

The two genes of the yeast *Saccharomyces cerevisiae*, PPX1 and PPN1, encode the polyphosphatases hydrolyzing polyP. The enzymes differ in substrate specificity, cellular localization, and role in polyP metabolism.

The expression and activity of these enzymes are interdependent and also depend on growth stage, P_i concentration, and carbon source.

The amino acid sequences of the two enzymes have no direct similarity. In higher eukaryotes, no direct orthologs of PPN1 are known, while PPX1 has a functionally related ortholog, the prune protein.

Our understanding of the role of these proteins in phosphorus metabolism is far from being complete but is crucial for understanding yeast phosphorus metabolism, as well as the mechanisms of human diseases and ontogenesis.

Acknowledgements The authors were supported by the Russian Foundation for Basic Research (grant 14-04-00515).

References

Andreeva NA, Okorokov LA (1993) Purification and characterization of highly active and stable polyphosphatase from *Saccharomyces cerevisiae* cell envelope. Yeast 9:127–139

Andreeva NA, Okorokov LA, Kulaev IS (1990) Purification and certain properties of cell envelope polyphosphatase of the yeast *Saccharomyces carlsbergensis*. Biochemistry (Mosc) 55:819–826

Andreeva NA, Kulakovskaya TV, Kulaev IS (1996) Purification and characterization of polyphosphatase from *Saccharomyces cerevisiae* cytosol. Biochemistry (Mosc) 61:1213–1220

Andreeva NA, Kulakovskaya TV, Kulaev IS (1998) Purification and properties of exopolyphosphatase isolated from *Saccharomyces cerevisiae* vacuoles. FEBS Lett 429:194–196

Andreeva NA, Kulakovskaya TV, Kulaev IS (2001) Two exopolyphosphatases of the cytosol of the yeast *S. cerevisiae*: comparative characteristics. Biochemistry (Mosc) 66:147–153

Andreeva NA, Kulakovskaya TV, Kulaev IS (2004) Purification and properties of exopolyphosphatase from the cytosol of *Saccharomyces cerevisiae* not encoded by the PPX1 gene. Biochemistry (Mosc) 69:387–393

Andreeva NA, Kulakovskaya TV, Kulaev IS (2006) High molecular mass exopolyphosphatase from the cytosol of the yeast *Saccharomyces cerevisiae* is encoded by the PPN1 gene. Biochemistry (Mosc) 71:975–977

Andreeva NA, Kulakovskaya TV, Kulakovskaya EV et al (2008) Polyphosphates and exopolyphosphatases in cytosol and mitochondria of *Saccharomyces cerevisiae* during growth on glucose or ethanol under phosphate surplus. Biochemistry (Mosc) 73:65–69

Andreeva N, Trilisenko L, Eldarov M et al (2015a) Polyphosphatase PPN1 of *Saccharomyces cerevisiae*: switching of exopolyphosphatase and endopolyphosphatase activities. PLoS One 10(3):e0119594. doi:10.1371/journal.pone.0119594

Andreeva N, Trilisenko L, Kulakovskaya T et al (2015b) Purification and properties of recombinant exopolyphosphatase PPN1 and effects of its overexpression on polyphosphate in *Saccharomyces cerevisiae*. J Biosci Bioeng 119:52–56

Carotenuto M, De Antonellis P, Liguori L et al (2014) H-Prune through GSK-3β interaction sustains canonical WNT/β-catenin signaling enhancing cancer progression in NSCLC. Oncotarget 5:5736–5749

Cunningham F, Amode MR, Barrell D et al (2015) Ensembl 2015. Nucleic Acids Res 43(Database issue):D662–D669. doi:10.1093/nar/gku1010

Duan RD (2006) Alkaline sphingomyelinase: an old enzyme with novel implications. Biochim Biophys Acta 1761:281–291

Eldarov MA, Baranov MV, Dumina MV et al (2013) Polyphosphates and exopolyphosphatase activities in the yeast *Saccharomyces cerevisiae* under overexpression of homologous and heterologous *PPN1* genes. Biochemistry (Mosc) 78:946–953

Forrest ARR, Kawaji H, Rehli M et al (2014) A promoter-level mammalian expression atlas. Nature 507:462–470. doi:10.1038/nature13182

Galasso A, Zollo M (2009) The Nm23-H1–h-Prune complex in cellular physiology: a 'tip of the iceberg' protein network perspective. Mol Cell Biochem 329:149–159

Goujon M, McWilliam H, Li W et al (2010) A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res 38(Web Server issue):W695–W699. doi:10.1093/nar/gkq313

Guranowski A, Starzynska E, Barnes LD et al (1998) Adenosine 5′-tetraphosphate phosphohydrolase activity is an inherent property of soluble exopolyphosphatase from *Saccharomyces cerevisiae*. Biochem Biophys Acta 1380:232–238

Kitasato T (1928) Über Metaphosphatase. Biochem Z 197:251–258

Kritsky MS, Chernysheva EK, Kulaev IS (1972) Polyphosphate depolymerase activity in cells of the fungus *Neurospora crassa* (in Russian). Biokhimiya 37:983–990

Kulaev IS, Vagabov VM, Kulakovskaya TV (2004) The biochemistry of inorganic polyphosphates. Wiley, Chichester

Kulakovskaya TV, Andreeva NA, Kulaev IS (1997) Adenosine-5′-tetraphosphate and guanosine-5′-tetraphosphate–new substrates of the cytosol exopolyphosphatase of *Saccharomyces cerevisiae*. Biochemistry (Mosc) 62:1180–1184

Kulakovskaya TV, Andreeva NA, Karpov AV et al (1999) Hydrolysis of tripolyphosphate by purified exopolyphosphatase of *Saccharomyces cerevisiae* cytosol: kinetic model. Biochemistry (Mosc) 64:990–993

Kulakovskaya TV, Andreeva NA, Trilisenko LV et al (2004) Two exopolyphosphatases in *Saccharomyces cerevisiae* cytosol at different culture conditions. Proc Biochem 39:1625–1630

Kulakovskaya TV, Trilisenko LV, Lichko LP et al (2006) The effect of inactivation of the exo-and endopolyphosphatase genes PPX1 and PPN1 on the level of different polyphosphates in the yeast *Saccharomyces cerevisiae*. Microbiology (Transl Mikrobiol, Russ) 75:25–28

Lichko LP, Kulakovskaya TV, Kulaev IS (1998) Membrane-bound and soluble polyphosphatases of mitochondria of *Saccharomycres cerevisiae*: identification and comparative characterization. Biochim Biophys Acta 1372:153–162

Lichko LP, Kulakovskaya TV, Kulaev IS (2000) Purification and characterization of a soluble polyphosphatase from mitochondria of *Saccharomyces cerevisiae*. Biochemistry (Mosc) 65:355–361

Lichko LP, Andreeva NA, Kulakovskaya TV et al (2003) Exopolyphosphatases of the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 3:233–238

Lichko LP, Kulakovskaya TV, Kulaev IS (2004a) Partial purification and characterization of nuclear exopolyphosphatase from *Saccharomyces cerevisiae* strain with inactivated PPX1 gene encoding a major yeast exopolyphosphatase. Biochemistry (Mosc) 69:270–274

Lichko L, Kulakovskaya T, Kulaev I (2004b) Inactivation of endopolyphosphatase gene PPN1 results in inhibition of expression of exopolyphosphatase PPX1 and high-molecular-mass exopolyphosphatase not encoded by PPX1 in *Saccharomyces cerevisiae*. Biochim Biophys Acta 1674:98–102

Lichko L, Kulakovskaya T, Pestov N et al (2006) Inorganic polyphosphates and exopolyphosphatases in cell compartments of the yeast *Saccharomyces cerevisiae* under inactivation of PPX1 and PPN1 genes. Biosci Rep 26:45–54

Lichko LP, Kulakovskaya TV, Kulakovskaya EV et al (2008) Inactivation of PPX1 and PPN1 genes encoding exopolyphosphatases of *Saccharomyces cerevisiae* does not prevent utilization of polyphosphates as phosphate reserve. Biochemistry (Mosc) 73:985–999

Lichko LP, Kulakovskaya TV, Kulaev IS (2010) Properties of partially purified endopolyphosphatase of the yeast Saccharomyces cerevisiae. Biochemistry (Mosc) 75:1404–1407

Lichko LP, MA Eldarov MA, MV Dumina MV et al (2014) PPX1 gene overexpression has no influence on polyphosphates in *Saccharomyces cerevisiae*. Biochemistry (Mosc) 79:1211–1215

Lonetti A, Szijgyarto Z, Bosch D et al (2011) Identification of an evolutionarily conserved family of inorganic polyphosphate endopolyphosphatases. J Biol Chem 286:31966–31974

Malmgren H (1952) Enzymatic breackdown of polymetaphosphate. V. Purification and specificity of the enzyme. Acta Chem Scand 6:16–26

Marchler-Bauer A, Derbyshire MK, Gonzales et al (2015) CDD: NCBI's conserved domain database. Nucleic Acids Res 43(Database issue):D222–D226. doi:10.1093/nar/gku1221

Marino N, Marchall JP, Steeg P (2011) Protein-protein interactions a mechanism regulating the antimetastatic properties of Nm23-H1. Naunyn-Schmiedeberg's Arch Pharmacol 384:351–362

Pestov NA, Kulakovskaya TV, Kulaev IS (2005) Effects of inactivation of the PPN1 gene on exopolyphosphatases, inorganic polyphosphates and function of mitochondria in the yeast *Saccharomyces cerevisiae*. FEMS Yeast Res 5:823–828

Sethuraman A, Rao NN, Kornberg A (2001) The endopolyphosphatase gene: essential in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 98:8542–8547

Shi X, Kornberg A (2005) Endopolyphosphatase in *Saccharomyces cerevisiae* undergoes post-translational activations to produce short-chain polyphosphates. FEBS Lett 579:2014–2018

Tammenkoski M, Moiseev VM, Lahti M et al (2007) Kinetic and mutational analyses of the major cytosolic exopolyphosphatase from *Saccharomyces cerevisiae*. J Biol Chem 282:9302–9311

Tammenkoski M, Koivula K, Cusanelli E et al (2008) Human metastasis regulator protein H-prune is a short-chain exopolyphosphatase. Biochemistry 47:9707–9713

Trilisenko LV, Kulakovskaya TV (2014) Polyphosphates as an energy source for growth of Saccharomyces cerevisiae. Biochemistry (Mosc) 79:478–482

Ugochukwu E, Lovering AL, Mather OC et al (2007) The crystal structure of the cytosolic exopolyphosphatase from *Saccharomyces cerevisiae* reveals the basis for substrate specificity. J Mol Biol 371:1007–1021

Wurst H, Kornberg A (1994) A soluble exopolyphosphatase of *Saccharomyces cerevisiae*. J Biol Chem 269:10996–101001

Wurst H, Shiba T, Kornberg A (1995) The gene for a major exopolyphosphatase of *Saccharomyces cerevisiae*. J Bacteriol 177:898–906

Polyphosphate Storage and Function in Acidocalcisomes

3

Roberto Docampo

Abstract

Bacterial and eukaryotic polyphosphate (polyP) accumulates in acidic vacuoles that have been named the *acidocalcisomes* to highlight their high calcium content together with the presence of other inorganic and organic cations. The large amount of polyP and cations in acidocalcisomes explains their high electron density when they are examined by electron microscopy. PolyP synthesis and translocation to the yeast acidocalcisome-like vacuole is catalyzed by the vacuolar transporter chaperone 4 (Vtc4), which is the catalytic subunit of the VTC complex and requires a proton gradient generated by the proton pump for this translocation. Similar VTC complexes have been found in acidocalcisomes of trypanosomatids and algae. Acidocalcisome polyP could be rapidly hydrolyzed under alkaline or hyposmotic stress. Hydrolysis of acidocalcisome polyP has been studied in trypanosomatids where it is catalyzed by an exopolyphosphatase (PPX) or by the exopolyphosphatase activity of a vacuolar soluble pyrophosphatase (VSP). It has been found that this hydrolytic process favors the release of osmolytes (phosphorus and cations) to the cytosol helping the regulatory volume decrease that follows hyposmotic stress. In some mammalian cells, like platelets and mast cells, acidocalcisome polyP is released into the circulation where it can have potent procoagulant, antifibrinolytic, and inflammatory actions and, under pathological conditions, could be involved in thrombosis. Fusion of acidocalcisomes with the contractile vacuole complex of *Chlamydomonas reinhardtii*, *Dictyostelium discoideum*, and *Trypanosoma cruzi* could also be involved in transfer of polyP or its hydrolytic products to the bladder to increase its osmolarity and ensuing water uptake necessary for its function. Finally, downregulation

R. Docampo
Department of Cellular Biology and Center for Tropical and Emerging Global Disease, 350A Paul D. Coverdell Center, University of Georgia, Athens, GA 30602, USA
e-mail: rdocampo@uga.edu

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_3

and upregulation of the expression of enzymes involved in polyP metabolism have revealed the role of polyP in growth, infectivity, and persistence of different parasites.

3.1 Introduction

Acidocalcisomes are acidic calcium stores rich in polyphosphate (polyP) (Patel and Docampo 2010). They have been conserved from bacteria to human cells and were originally named after their discovery in trypanosomatids (Vercesi et al. 1994; Docampo et al. 1995, 2005). Their acidity is maintained in bacteria and protists by either a vacuolar H^+-pyrophosphatase or a vacuolar H^+-ATPase or both, while in other eukaryotic cells a V-H^+-ATPase is required (Docampo and Moreno 2011). Acidocalcisomes of *D. discoideum* (Marchesini et al. 2002), trypanosomatids (Lu et al. 1998; Luo et al. 2004), and *Toxoplasma gondii* (Luo et al. 2005) possess a Ca^{2+}-ATPase for Ca^{2+} uptake. Ca^{2+} release from some acidocalcisomes, like those of *Trypanosoma brucei*, is through a Ca^{2+} channel, the inositol 1,4,5-trisphosphate receptor (Huang et al. 2013). Because polyP is a polyanion, its charges are balanced by several organic (Rohloff et al. 2003) and inorganic (Scott et al. 1997) cations, and acidocalcisomes possess transporters for these cations that have been identified in recent proteomic studies (Huang et al. 2014). The function of acidocalcisomes is related to the function of its components, among them polyP. Figure 3.1 shows a scheme of the pumps, channels, and transporters present in an acidocalcisome of *T. brucei* and Table 3.1 shows the species in which acidocalcisomes have been studied. In this chapter we will limit our discussion to what is known about eukaryotic acidocalcisomes, which have been studied in more detail.

3.2 Polyphosphate Content of Acidocalcisomes

Acidocalcisomes possess large amounts of phosphorus compounds, such as phosphate, pyrophosphate (PP_i), and short- and long-chain polyP (Docampo and Moreno 2011). Some trypanosomatids have millimolar cellular levels of polyP, most of it concentrated in acidocalcisomes (Table 3.2) (Ruiz et al. 2001a; Rodrigues et al. 2002a). As acidocalcisomes occupy in these cells only 1–2 % of their volume (Miranda et al. 2000), their concentration inside the organelles could reach molar levels, in terms of phosphate units (Docampo et al. 2005). This is in agreement with the high electron density of the organelles when observed by electron microscopy (Fig. 3.2a) and also their high density upon ultracentrifugation, which allows their purification from other subcellular fractions (Scott et al. 1997; Rodrigues et al. 1999a, b; Scott and Docampo 2000; Rohloff et al. 2011; Huang et al. 2014). Very short chain polyP such as $polyP_3$, $polyP_4$, and $polyP_5$ can be easily detected by ^{31}P-NMR spectrometry (Moreno et al. 2000) (Fig. 3.3), while longer chain polyP (up to ~700 or more orthophosphate units) can be detected using biochemical techniques

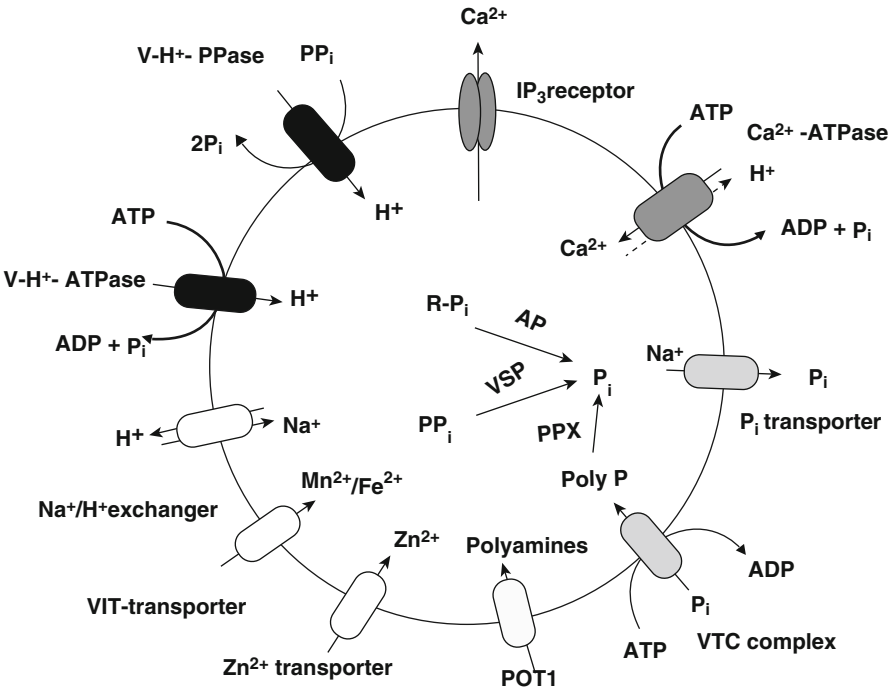

Fig. 3.1 Scheme of an acidocalcisome of procyclic stages of *T. brucei*. Ca^{2+} uptake is by a Ca^{2+}-ATPase and Ca^{2+} release is by the inositol 1,4,5-trisphosphate receptor (IP_3 receptor) (in *dark gray*). H^+ transport is by the vacuolar H^+-PPase (V-H^+-PPase) or the vacuolar H^+-ATPase (V-H^+-ATPase) (in *black*). A cation exchanger (Na^+/H^+ exchanger), cation transporters (VIT-transporter, Zn^{2+} transporter), and a polyamine transporter (POT1) transport inorganic and organic cations (in *white*). A vacuolar transporter chaperone complex (VTC) synthesizes polyP using ATP and translocates it into the organelle and a Na^+/P_i symporter releases Na^+ and P_i from acidocalcisomes (in *light gray*). Within acidocalcisomes there is a vacuolar soluble PPase (*VSP*), an exopolyphosphatase (*PPX*), and an acid phosphatase (*AP*) (Modified from Huang et al. 2014)

(Ruiz et al. 2001a). The most useful technique is based on the hydrolysis of polyP by yeast exopolyphosphatase (PPX) and determination of the phosphorus released (Ruiz et al. 2001a). SDS gel electrophoresis is also very useful to detect the size distribution of polyP in cells (Fig. 3.4a) (Ruiz et al. 2001a; Ramos et al. 2010a, b; Lander et al. 2013), and 4′-6′-diamino-2-phenylindole (DAPI) staining is ideal to detect its cellular localization (Fang et al. 2007b; Ramos et al. 2010a, b). Figure 3.4b, for example, shows staining of acidocalcisomes within larger vacuoles of the chicken egg yolk, a structure known as compound organelle (Ramos et al. 2010b). It has been possible to do immunolocalization studies using the epitope-tagged polyphosphate binding domain (PPBD) of *E. coli* exopolyphosphatase to localize polyP in some acidocalcisomes (Ramos et al. 2010a; Moreno-Sanchez et al. 2012). It is also possible to detect polyP using magic-angle spinning ^{31}P-NMR techniques with isolated acidocalcisomes (Moreno et al. 2002). When the organelles are

Table 3.1 Organisms and cells in which acidocalcisomes have been investigated in more detail

Cells or organisms	Selected references
Trypanosomatids	
Trypanosoma cruzi	Docampo et al. (1995), Scott et al. (1997), Lu et al. (1998), Scott and Docampo (2000), Miranda et al. (2000), Ruiz et al. (2001a), Montalvetti et al. (2004), Rohloff et al. (2004), Fang et al. (2007b), de Jesus et al. (2010), Li et al. (2011), Galizzi et al. (2013), Ulrich et al. (2014), Niyogi et al. (2015)
Trypanosoma brucei	Vercesi et al. (1994), Rodrigues et al. (1999a), Luo et al. (2004), Lemercier et al. (2004), Fang et al. (2007a), Huang et al. (2013, 2014), Lander et al. (2013), Ulrich et al. (2014), Li and He (2014)
Trypanosoma evansi	Mendoza et al. (2002)
Leishmania donovani	Rodrigues et al. (1999b), Sahin et al. (2008)
Leishmania major	Rodrigues et al. (2002a), Moreno et al. (2000, 2002), Zhang et al. (2005), Besteiro et al. (2008), Madeira da Silva and Beverley (2010)
Leishmania amazonensis, Phytomonas françai and other monogenetic trypanosomatids	Miranda et al. (2004a, b, c), Espiau et al. (2006)
Apicomplexan	
Toxoplasma gondii	Moreno and Zhong (1996), Luo et al. (2001), Rodrigues et al. (2002b), Luo et al. (2005), Rohloff et al. (2011)
Plasmodium falciparum	Ruiz et al. (2004c)
Eimeria tenella	Soares Medeiros et al. (2011)
Eimeria acervulina	Soares Medeiros et al. (2011)
Algae	
Chlamydomonas reinhardtii	Ruiz et al. (2001b), Aksoy et al. (2014), Hong-Hermesdorf et al. (2014)
Cyanidioschyzon merolae	Yagisawa et al. (2009)
Slime mold	
Dictyostelium discoideum	Marchesini et al. (2002)
Bacteria	
Agrobacterium tumefaciens	Seufferheld et al. (2003)
Rhodospirillum rubrum	Seufferheld et al. (2004)
Eggs	
Insect	Motta et al. (2009), Ramos et al. (2011)
Sea urchin	Ramos et al. (2010a)
Chicken	Ramos et al. (2010b)
Human	
Platelets	Ruiz et al. (2004a)
Mast cells and basophils	Moreno-Sanchez et al. (2012)

Table 3.2 Polyphosphate levels in trypanosomatids (mM)

PolyP size	*T. cruzi* epimastigotes	*T. cruzi* amastigotes	*T. cruzi* trypomastigotes	*L. major* promastigotes
Short chain	54.3 ± 0.3	25.5 ± 5.1	3.1 ± 1.4	21.4 ± 3.0
Long chain	2.89 ± 0.29	0.13 ± 0.01	0.82 ± 0.005	55.9 ± 5.6

T. cruzi results are from Scott et al. (1997) and *L. major* results are from Rodrigues et al. (2002a)

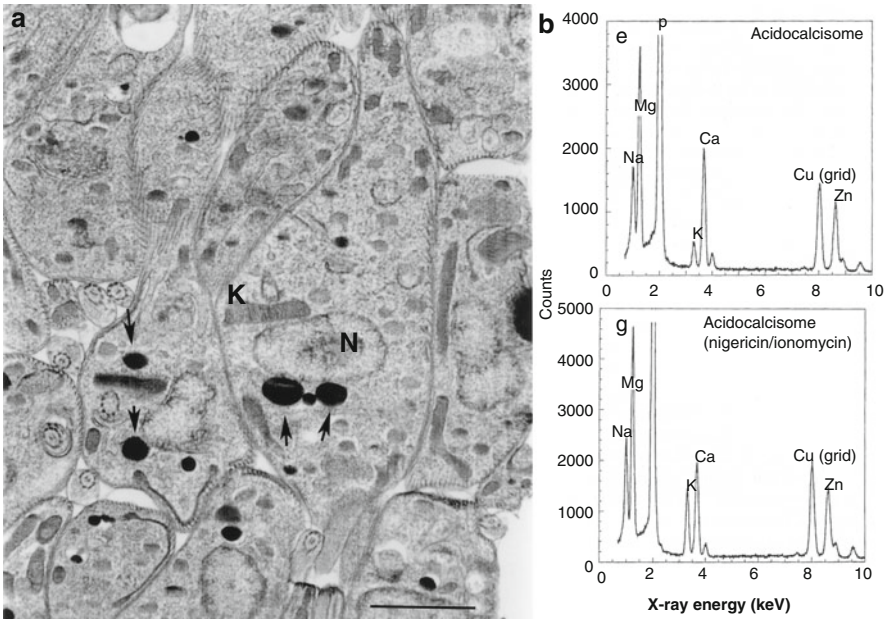

Fig. 3.2 Transmission electron microscopy and X-ray spectra of acidocalcisomes of *T. cruzi*. (**a**) Unstained cells were prepared by quick freezing and ultramicrotomy (Scott et al. 1997). The electron-dense vacuoles (*arrows*) correspond to acidocalcisomes. *N* nucleus, *K* kinetoplast. Bar = 1 μm. (**b**) X-ray spectra recorded from the electron-dense organelles (acidocalcisomes). Only major Kα peaks are labeled (*Na*, *Mg*, *P*, *K*, *Ca*, and *Zn*). The Cu peak originates from spurious excitation of the specimen support grid. The acidocalcisomes from samples treated with ionomycin/nigericin showed increase in the K peak suggesting that they are acidic (This research was originally published in Scott et al. (1997). © the American Society for Biochemistry and Molecular Biology)

isolated, they lose their acidity because ATP and PP_i are not available for the proton pumps to function, and polyP and cations condense to solid state to give ^{31}P-NMR spectra showing predominance of α phosphates (Moreno et al. 2002). The results suggest that the very short chain class of polyP must be dominated by tripolyphosphate ($polyP_3$) and tetrapolyphosphate ($polyP_4$). In fact, these spectra have similarities with that of the model compound disodium, dicalcium tripolyphosphate

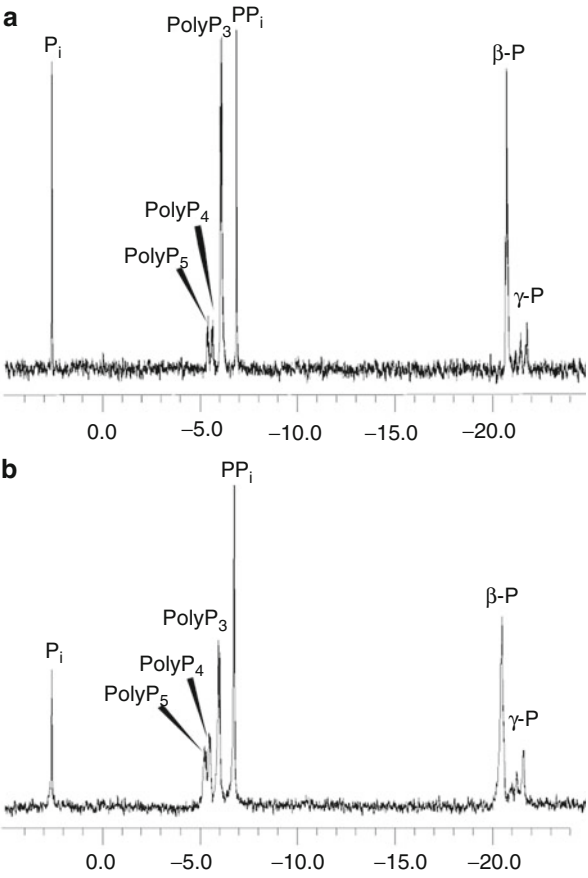

Fig. 3.3 ^{31}P-NMR spectra of acidocalcisomes. (**a**, **b**) 303.6-MHz spectrum (^{1}H decoupled) of perchloric acid extracts of isolated acidocalcisomes from procyclic (**a**) and bloodstream (**b**) trypomastigotes of *T. brucei*, showing peaks corresponding to P_i; the α phosphates of pentapolyphosphate ($polyP_5$), tetrapolyphosphate ($polyP_4$), tripolyphosphate ($polyP_3$); PP_i; the β phosphates of tri-, tetra-, and pentapolyphosphate (β-P); and the γ phosphate (central) of pentapolyphosphate (γ-P) (This research was originally published in Moreno et al. (2000). © the American Society for Biochemistry and Molecular Biology)

(Moreno et al. 2002) (Fig. 3.5). PolyP negative charges are compensated by the abundant presence of organic (basic amino acids (Rohloff et al. 2003) and probably polyamines) and inorganic (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Na^{+}, K^{+}) cations (Docampo and Moreno 2011). Inorganic cations are easily detected by X-ray microanalysis of intact acidocalcisomes (Scott et al. 1997) (Fig. 3.2b). How the structure of this complex polymer is maintained within acidocalcisomes is not yet known. Alkalinization or hyposmotic treatment of cells is known to result in hydrolysis of acidocalcisome polyP (Ruiz et al. 2001a).

3.3 Synthesis and Degradation of Acidocalcisome PolyP

D. discoideum has a polyphosphate kinase (DdPPK1) that is an orthologue of the bacterial PPK1 (Zhang et al. 2007), and that was probably acquired by horizontal transfer in this and other early eukaryotes (Whitehead et al. 2013). However, most eukaryotes lack orthologues to bacterial PPK1 and PPK2. *D. discoideum* possesses

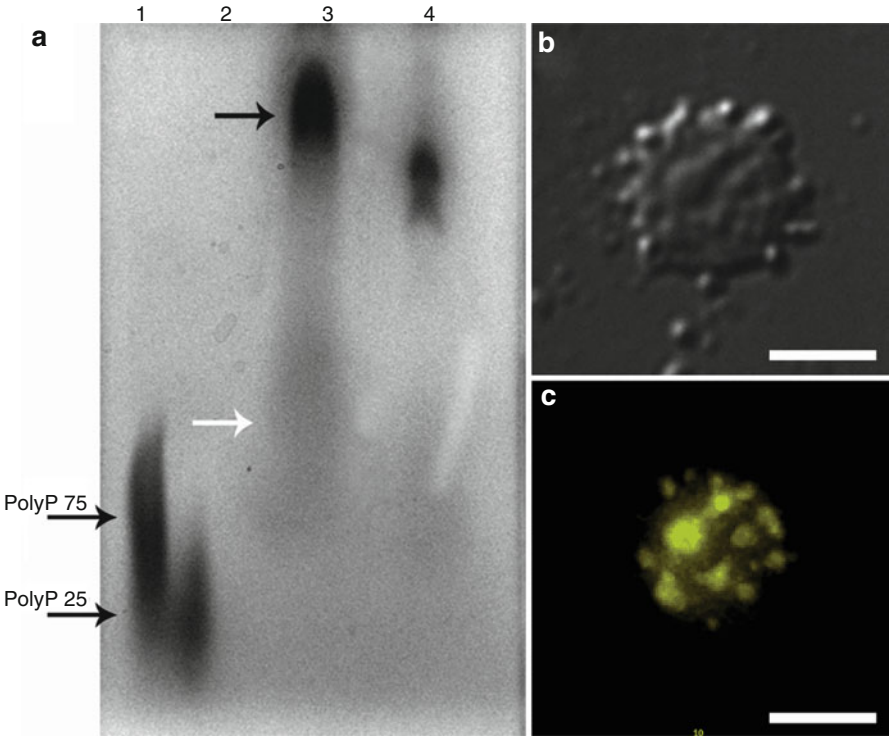

Fig. 3.4 Detection of polyP in the yolk of chicken eggs and its localization to acidocalcisomes. (**a**) PolyP was extracted from the yolk organelles, separated by agarose gel electrophoresis and stained with toluidine blue. Synthetic polyP (5 μg) with mean chain lengths of 25 ($polyP_{25}$) or 75 ($polyP_{75}$) phosphate units were loaded onto lanes 1 and 2 as size standards *(arrows)*. Lane 3: polyP from yolk organelles (the *black arrow* indicates high molecular mass polyP and the *white arrow* indicates shorter polyP). Lane 4, polyP from yolk organelles was incubated for 30 min with recombinant yeast PPX prior to electrophoresis. (**b**, **c**) DIC (differential interference contrast) and DAPI staining of compound organelles demonstrate poly P localization in internal vesicles. Scale bars, 5 μm (Reprinted from Ramos et al. (2010a) with permission, copyright (2010), with permission from Elsevier)

a second polyP kinase (DdPPK2) that was reported as composed of three subunits that are actin-related proteins (Gomez-Garcia and Kornberg 2004). Both enzymes were found in vacuoles although it is not known whether these vacuoles correspond to the acidocalcisomes described in these organisms (Marchesini et al. 2002). DdPPK1 is apparently the most important synthetic enzyme and depletion of polyP after knockout of its gene affects spore germination and energy metabolism (Livermore et al. 2016).

A significant breakthrough in the field was the discovery, in yeast, that the vacuolar transporter chaperone 4 (Vtc4) was a polyP polymerase using ATP to form polyP (Hothorn et al. 2009). The vacuolar transporter chaperone complex (VTC) is composed of four members (Vtc1-Vtc4) that form hetero-oligomer complexes

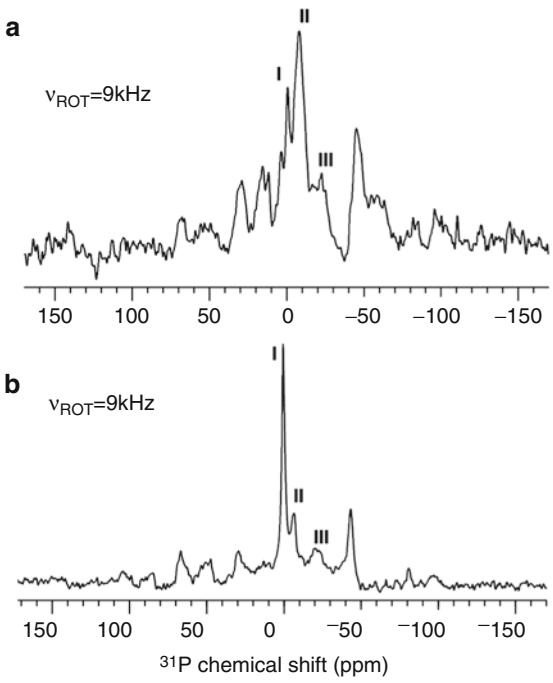

Fig. 3.5 ^{31}P-NMR spectra of intact acidocalcisomes isolated from *T. brucei* bloodstream forms (**a**) and *L. major* promastigotes (**b**). Resonance I was attributed to inorganic phosphate and headgroup phosphates of acidocalcisome membrane lipids. Resonance II was assigned to terminal α-phosphates of polyP, including PP_i while resonance III was assigned to the β-phosphates of polyP (Reprinted from Moreno et al. (2002), copyright (2002), with permission from Elsevier)

(Vtc1,2,4 and VTc1,3,4), and *null* mutants of several subunits of the complex were reported to have decreased polyP levels in yeast (Ogawa et al. 2000). Vtc4 was discovered as the catalytic subunit of the complex that catalyzes the synthesis and translocation of polyP to the inside of the yeast vacuole (Hothorn et al. 2009; Gerasimaite et al. 2014) requiring a proton gradient generated by the vacuolar H^+-ATPase for this translocation (Gerasimaite et al. 2014). Subunits of this complex were also found in *T. brucei* (Fang et al. 2007; Lander et al. 2013; Huang et al. 2014), *T. cruzi* (Ulrich et al. 2014), *T. gondii* (Rooney et al. 2011), *C. reinhardtii* (Aksoy et al. 2014), and *Cyanidioschyzon merolae* (Yagisawa et al. 2009). Vtc4 was localized to acidocalcisomes in many of these organisms, and its catalytic activity was demonstrated in trypanosomes where it is essential for growth and infectivity (Lander et al. 2013). However, conditional mutants for TbVtc4 were still capable of synthesizing some polyP suggesting that other biosynthetic pathways might be present.

It has been shown that yeast deficient in phospholipase C, which converts phosphatidylinositol 4,5-diphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3), as well as in other enzymes of the inositol pyrophosphate pathway: inositol phosphate multikinase, IPMK, that converts IP_3 into IP_4 and IP_5, and inositol hexakisphosphate kinase, IP6K, which converts IP_6 into IP_7, lacks polyP, suggesting a metabolic link between these pathways (Lonetti et al. 2011). Similar results were obtained in platelets from *IP6K*$^{-/-}$ mice that had a significant depletion of polyP from the

acidocalcisome-like dense granules, and this was associated to blood coagulation defects (Ghosh et al. 2013). This is because polyP is released from dense granules (acidocalcisomes) by activated platelets (Ruiz et al. 2004a) and promotes blood coagulation (Smith et al. 2006).

Degradation of polyP in eukaryotes is catalyzed by exopolyphosphatases (PPXs) and endopolyphosphatases (PPNs). An exopolyphosphatase activity was initially detected in acidocalcisome fractions of *T. cruzi* (Ruiz et al. 2001a), and an exopolyphosphatase was localized to the acidocalcisomes and cytosol of *Leishmania major* (Rodrigues et al. 2002a). Trypanosomatids also possess a vacuolar soluble pyrophosphatase (VSP) that also partially localizes to acidocalcisomes and cytosol and can hydrolyze pyrophosphate (PP_i) or polyP depending on the presence of Mg^{2+} (pyrophosphatase activity) or transition metals such as Zn^{2+}, Mn^{2+}, and Co^{2+} (exopolyphosphatase activity) (Lemercier et al. 2004; Espiau et al. 2006; Galizzi et al. 2013). This enzyme has two distinct regions, an N-terminal containing an EF-hand domain and a C-terminal containing the catalytic domain (Lemercier et al. 2004). The exopolyphosphatase but not the pyrophosphatase activity of the enzyme is inhibited by PP_i analogs (bisphosphonates) (Kotsikorou et al. 2005), and since it is essential in trypanosomatids (Lemercier et al. 2004; Espiau et al. 2006), it is a potential drug target. Interestingly, overexpression of this enzyme in *T. cruzi* resulted in decreased levels of PP_i and polyP, less responsiveness of the cells to osmotic stress, and less persistence of the parasite in mice tissues, suggesting that PP_i and polyP are required for the parasite to resist the stressful conditions of its host (Galizzi et al. 2013).

Although endopolyphosphatases have been described in yeast (Sethuraman et al. 2001; Shi and Kornberg 2005; Lonetti et al. 2011) and mammalian cells (Lonetti et al. 2011), their localization has not been studied and we cannot rule out their potential localization in acidocalcisomes or acidocalcisome-like vacuoles.

3.4 Functions of Acidocalcisome PolyP

The functions of acidocalcisome polyP in eukaryotic cells are starting to be defined. An important discovery was that polyP accumulated in acidocalcisomes from human platelets (dense granules) can be secreted when platelets are stimulated by thrombin (Ruiz et al. 2004a; Muller et al. 2009), ADP, or collagen (Muller et al. 2009). This released polyP was found to have procoagulant and antifibrinolytic actions and remain in circulation for considerable time (Smith et al. 2006). PolyP acts at four points in the blood-clotting cascade: initiation of the contact pathway, factor V activation, fibrin polymerization, and factor XI back-activation by thrombin (Morrissey et al. 2012). Activation of factor XII to XIIa by polyP leads to bradykinin formation by kallikrein-mediated high molecular weight kininogen cleavage (Muller et al. 2009). Bradykinin stimulates kinin B2 receptor activating various intracellular signaling pathways that lead to inflammatory reactions (Muller et al. 2009). PolyP is also present in serotonin-containing granules of mast cells and in basophils and is also released by IgE-stimulated mast cells (Moreno-Sanchez et al. 2012). These results could explain the procoagulant (Muller et al. 1993) and

pro-inflammatory (Oschatz et al. 2011) roles of mast cells. PolyP in blood could be a target for antithrombotic agents (Docampo 2014).

Acidocalcisome polyP has also a role in storage of organic and inorganic cations, which neutralize its anionic charges within the organelles and are released or taken up when polyP is hydrolyzed or synthesized, respectively. This ability is relevant during cellular hyposmotic and hyperosmotic stresses. Hydrolysis of polyP, which occurs under hyposmotic stress, releases osmolytes (phosphorus, cations) increasing the osmolarity of the medium, while synthesis of polyP, which occurs under hyperosmotic stress, stimulates osmolyte uptake and therefore reduction in the cytosol osmolarity (Ruiz et al. 2001a). These changes help the cells recover their initial volume after osmotic stress. Acidocalcisomes also increase their volume under hyposmotic conditions (Li et al. 2011) and fuse with the contractile vacuole in several protists (Ruiz et al. 2001b; Marchesini et al. 2002; Rohloff et al. 2004; Niyogi et al. 2015). A model has been postulated where this fusion is accompanied by the release of osmolytes to the bladder of the contractile vacuole attracting water that is then expelled to the extracellular medium (Docampo et al. 2013). The osmolytes (cations and phosphorus) would then return to the cytosol as only water is expelled (Docampo et al. 2013). This model is in agreement with the presence of a phosphate transporter in the contractile vacuole bladder (Jimenez and Docampo 2015), which could return phosphorus to the cytosol. The role of acidocalcisome polyP in osmoregulation is also supported by reports showing that altering the expression of enzymes involved in their synthesis and degradation affects the morphology of acidocalcisomes and their response to osmotic stress (Lemercier et al. 2004; Fang et al. 2007a; Galizzi et al. 2013; Lander et al. 2013).

Finally, conditions that result in decreased levels of acidocalcisome polyP have been associated to a reduction in pathogenicity of several microorganisms such as *T. brucei* (Fang et al. 2007a; Lander et al. 2013), *T. cruzi* (Galizzi et al. 2013), *T. gondii* (Luo et al. 2005), and *L. major* (Zhang et al. 2005). This effect could be attributed to either increased osmotic fragility of the parasites or to a role of polyP in modulating the host immune response.

Acknowledgments Work in the author's laboratory is supported by the US National Institutes of Health grants AI107663 and AI077538.

References

Aksoy M, Pootakham W, Grossman AR (2014) Critical function of a *Chlamydomonas reinhardtii* putative polyphosphate polymerase subunit during nutrient deprivation. Plant Cell 26(10):4214–4229. doi:10.1105/tpc.114.129270

Besteiro S, Tonn D, Tetley L et al (2008) The AP3 adaptor is involved in the transport of membrane proteins to acidocalcisomes of *Leishmania*. J Cell Sci 121(Pt 5):561–570

de Jesus TC, Tonelli RR, Nardelli SC et al (2010) Target of rapamycin (TOR)-like 1 kinase is involved in the control of polyphosphate levels and acidocalcisome maintenance in *Trypanosoma brucei*. J Biol Chem 285(31):24131–24140. doi:10.1074/jbc.M110.120212

Docampo R (2014) Polyphosphate: a target for thrombosis attenuation. Blood 124(22):3177–3178. doi:10.1182/blood-2014-09-601641

Docampo R, Moreno SN (2011) Acidocalcisomes. Cell Calcium 50(2):113–119. doi:10.1016/j.ceca.2011.05.012

Docampo R, Scott DA, Vercesi AE et al (1995) Intracellular Ca^{2+} storage in acidocalcisomes of *Trypanosoma cruzi*. Biochem J 310(Pt 3):1005–1012

Docampo R, de Souza W, Miranda K et al (2005) Acidocalcisomes – conserved from bacteria to man. Nat Rev Microbiol 3(3):251–261. doi:10.1038/nrmicro1097

Docampo R, Jimenez V, Lander N et al (2013) New insights into roles of acidocalcisomes and contractile vacuole complex in osmoregulation in protists. Int Rev Cell Mol Biol 305:69–113. doi:10.1016/B978-0-12-407695-2.00002-0

Espiau B, Lemercier G, Ambit A et al (2006) A soluble pyrophosphatase, a key enzyme for polyphosphate metabolism in *Leishmania*. J Biol Chem 281(3):1516–1523. doi:10.1074/jbc.M506947200

Fang J, Rohloff P, Miranda K et al (2007a) Ablation of a small transmembrane protein of *Trypanosoma brucei* (TbVTC1) involved in the synthesis of polyphosphate alters acidocalcisome biogenesis and function, and leads to a cytokinesis defect. Biochem J 407(2):161–170

Fang J, Ruiz FA, Docampo M et al (2007b) Overexpression of a Zn^{2+}-sensitive soluble exopolyphosphatase from *Trypanosoma cruzi* depletes polyphosphate and affects osmoregulation. J Biol Chem 282(44):32501–32510. doi:10.1074/jbc.M704841200

Galizzi M, Bustamante JM, Fang J et al (2013) Evidence for the role of vacuolar soluble pyrophosphatase and inorganic polyphosphate in *Trypanosoma cruzi* persistence. Mol Microbiol 90(4):699–715. doi:10.1111/mmi.12392

Gerasimaite R, Sharma S, Desfougeres Y et al (2014) Coupled synthesis and translocation restrains polyphosphate to acidocalcisome-like vacuoles and prevents its toxicity. J Cell Sci 127(23):5093–5104. doi:10.1242/jcs.159772

Ghosh S, Shukla D, Suman K et al (2013) Inositol hexakisphosphate kinase 1 maintains hemostasis in mice by regulating platelet polyphosphate levels. Blood 122(8):1478–1486. doi:10.1182/blood-2013-01-481549

Gomez-Garcia MR, Kornberg A (2004) Formation of an actin-like filament concurrent with the enzymatic synthesis of inorganic polyphosphate. Proc Natl Acad Sci U S A 101(45):15876–15880. doi:10.1073/pnas.0406923101

Hong-Hermesdorf A, Miethke M, Gallaher SD et al (2014) Subcellular metal imaging identifies dynamic sites of Cu accumulation in *Chlamydomonas*. Nat Chem Biol 10(12):1034–1042. doi:10.1038/nchembio.1662

Hothorn M, Neumann H, Lenherr ED et al (2009) Catalytic core of a membrane-associated eukaryotic polyphosphate polymerase. Science 324(5926):513–516. doi:10.1126/science.1168120

Huang G, Bartlett PJ, Thomas AP et al (2013) Acidocalcisomes of *Trypanosoma brucei* have an inositol 1,4,5-trisphosphate receptor that is required for growth and infectivity. Proc Natl Acad Sci U S A 110(5):1887–1892. doi:10.1073/pnas.1216955110

Huang G, Ulrich PN, Storey M et al (2014) Proteomic analysis of the acidocalcisome, an organelle conserved from bacteria to human cells. PLoS Pathog 10(12):e1004555. doi:10.1371/journal.ppat.1004555

Jimenez V, Docampo R (2015) TcPho91 is a contractile vacuole phosphate sodium symporter that regulates phosphate and polyphosphate metabolism in *Trypanosoma cruzi*. Mol Microbiol 97(5):911–925. doi:10.1111/mmi.13075

Kotsikorou E, Song Y, Chan JM et al (2005) Bisphosphonate inhibition of the exopolyphosphatase activity of the *Trypanosoma brucei* soluble vacuolar pyrophosphatase. J Med Chem 48(19):6128–6139. doi:10.1021/jm058220g

Lander N, Ulrich PN, Docampo R (2013) *Trypanosoma brucei* vacuolar transporter chaperone 4 (TbVtc4) is an acidocalcisome polyphosphate kinase required for in vivo infection. J Biol Chem 288(47):34205–34216. doi:10.1074/jbc.M113.518993

Lemercier G, Espiau B, Ruiz FA et al (2004) A pyrophosphatase regulating polyphosphate metabolism in acidocalcisomes is essential for *Trypanosoma brucei* virulence in mice. J Biol Chem 279(5):3420–3425. doi:10.1074/jbc.M309974200

Li FJ, He CY (2014) Acidocalcisome is required for autophagy in *Trypanosoma brucei*. Autophagy 10(11):1978–1988. doi:10.4161/auto.36183

Li ZH, Alvarez VE, De Gaudenzi JG et al (2011) Hyperosmotic stress induces aquaporin-dependent cell shrinkage, polyphosphate synthesis, amino acid accumulation, and global gene expression changes in *Trypanosoma cruzi*. J Biol Chem 286(51):43959–43971. doi:10.1074/jbc.M111.311530

Livermore TM, Chubb JR, Saiardi A (2016) Developmental accumulation of inorganic polyphosphate affects germination and energetic metabolism *in Dictyostelium discoideum*. Proc Natl Acad Sci U S A 113(4):996–1001. doi:10.1073/pnas.1519440113

Lonetti A, Szijgyarto Z, Bosch D et al (2011) Identification of an evolutionarily conserved family of inorganic polyphosphate endopolyphosphatases. J Biol Chem 286(37):31966–31974. doi:10.1074/jbc.M111.266320

Lu HG, Zhong L, de Souza W et al (1998) Ca^{2+} content and expression of an acidocalcisomal calcium pump are elevated in intracellular forms of *Trypanosoma cruzi*. Mol Cell Biol 18(4):2309–2323

Luo S, Vieira M, Graves J et al (2001) A plasma membrane-type Ca^{2+}-ATPase co-localizes with a vacuolar H^+-pyrophosphatase to acidocalcisomes of *Toxoplasma gondii*. Embo J 20(1–2):55–64. doi:10.1093/emboj/20.1.55

Luo S, Rohloff P, Cox J et al (2004) *Trypanosoma brucei* plasma membrane-type Ca^{2+}-ATPase 1 (*TbPMC1*) and 2 (*TbPMC2*) genes encode functional Ca^{2+}-ATPases localized to the acidocalcisomes and plasma membrane, and essential for Ca^{2+} homeostasis and growth. J Biol Chem 279(14):14427–14439. doi:10.1074/jbc.M309978200

Luo S, Ruiz FA, Moreno SN (2005) The acidocalcisome Ca^{2+}-ATPase (TgA1) of *Toxoplasma gondii* is required for polyphosphate storage, intracellular calcium homeostasis and virulence. Mol Microbiol 55(4):1034–1045. doi:10.1111/j.1365-2958.2004.04464.x

Madeira da Silva L, Beverley SM (2010) Expansion of the target of rapamycin (TOR) kinase family and function in *Leishmania* shows that TOR3 is required for acidocalcisome biogenesis and animal infectivity. Proc Natl Acad Sci U S A 107(26):11965–11970. doi:10.1073/pnas.1004599107

Marchesini N, Ruiz FA, Vieira M et al (2002) Acidocalcisomes are functionally linked to the contractile vacuole of *Dictyostelium discoideum*. J Biol Chem 277(10):8146–8153. doi:10.1074/jbc.M111130200

Mendoza M, Mijares A, Rojas H et al (2002) Physiological and morphological evidences for the presence acidocalcisomes in *Trypanosoma evansi*: single cell fluorescence and ^{31}P NMR studies. Mol Biochem Parasitol 125(1–2):23–33

Miranda K, Benchimol M, Docampo R et al (2000) The fine structure of acidocalcisomes in *Trypanosoma cruzi*. Parasitol Res 86(5):373–384

Miranda K, Docampo R, Grillo O et al (2004a) Dynamics of polymorphism of acidocalcisomes in *Leishmania* parasites. Histochem Cell Biol 121(5):407–418. doi:10.1007/s00418-004-0646-4

Miranda K, Rodrigues CO, Hentchel J et al (2004b) Acidocalcisomes of *Phytomonas francai* possess distinct morphological characteristics and contain iron. Microsc Microanal 10(5):647–655. doi:10.1017/S1431927604040887

Miranda K, Docampo R, Grillo O et al (2004c) Acidocalcisomes of trypanosomatids have species-specific elemental composition. Protist 155(4):395–405. doi:10.1078/1434461042650361

Montalvetti A, Rohloff P, Docampo R (2004) A functional aquaporin co-localizes with the vacuolar proton pyrophosphatase to acidocalcisomes and the contractile vacuole complex of *Trypanosoma cruzi*. J Biol Chem 279(37):38673–38682. doi:10.1074/jbc.M406304200

Moreno SN, Zhong L (1996) Acidocalcisomes in *Toxoplasma gondii* tachyzoites. Biochem J 313(Pt 2):655–659

Moreno B, Urbina JA, Oldfield E et al (2000) ^{31}P NMR spectroscopy of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. Evidence for high levels of condensed inorganic phosphates. J Biol Chem 275(37):28356–28362. doi:10.1074/jbc.M003893200

Moreno B, Rodrigues CO, Bailey BN et al (2002) Magic-angle spinning ^{31}P NMR spectroscopy of condensed phosphates in parasitic protozoa: visualizing the invisible. FEBS Lett 523(1–3):207–212

Moreno-Sanchez D, Hernandez-Ruiz L, Ruiz FA et al (2012) Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. J Biol Chem 287(34):28435–28444. doi:10.1074/jbc.M112.385823

Morrissey JH, Choi SH, Smith SA (2012) Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation. Blood 119(25):5972–5979. doi:10.1182/blood-2012-03-306605

Motta LS, Ramos IB, Gomes FM et al (2009) Proton-pyrophosphatase and polyphosphate in acidocalcisome-like vesicles from oocytes and eggs of *Periplaneta americana*. Insect Biochem Mol Biol 39(3):198–206. doi:10.1016/j.ibmb.2008.11.003

Muller AD, Lichtenbeld HH, Blijham GH et al (1993) Mast cell lysate induces a procoagulant effect on endothelial cells. Thromb Res 72(3):263–267

Muller F, Mutch NJ, Schenk WA et al (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell 139(6):1143–1156. doi:10.1016/j.cell.2009.11.001

Niyogi S, Jimenez V, Girard-Dias W et al (2015) Rab32 is essential for maintaining functional acidocalcisomes and for growth and infectivity of *Trypanosoma cruzi*. J Cell Sci 128:2363–2373. doi:10.1242/jcs.169466

Ogawa N, DeRisi J, Brown PO (2000) New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol Biol Cell 11(12):4309–4321

Oschatz C, Maas C, Lecher B et al (2011) Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo. Immunity 34(2):258–268. doi:10.1016/j.immuni.2011.02.008

Patel S, Docampo R (2010) Acidic calcium stores open for business: expanding the potential for intracellular Ca^{2+} signaling. Trends Cell Biol 20(5):277–286. doi:10.1016/j.tcb.2010.02.003

Ramos IB, Miranda K, Pace DA et al (2010a) Calcium- and polyphosphate-containing acidic granules of sea urchin eggs are similar to acidocalcisomes, but are not the targets for NAADP. Biochem J 429(3):485–495. doi:10.1042/BJ20091956

Ramos IB, Miranda K, Ulrich P et al (2010b) Calcium- and polyphosphate-containing acidocalcisomes in chicken egg yolk. Biol Cell 102(7):421–434. doi:10.1042/BC20100011

Ramos I, Gomes F, Koeller CM et al (2011) Acidocalcisomes as calcium- and polyphosphate-storage compartments during embryogenesis of the insect *Rhodnius prolixus* Stahl. PLoS One 6(11):e27276. doi:10.1371/journal.pone.0027276

Rodrigues CO, Scott DA, Docampo R (1999a) Characterization of a vacuolar pyrophosphatase in *Trypanosoma brucei* and its localization to acidocalcisomes. Mol Cell Biol 19(11):7712–7723

Rodrigues CO, Scott DA, Docampo R (1999b) Presence of a vacuolar H+-pyrophosphatase in promastigotes of *Leishmania donovani* and its localization to a different compartment from the vacuolar H^+-ATPase. Biochem J 340(Pt 3):759–766

Rodrigues CO, Ruiz FA, Vieira M et al (2002a) An acidocalcisomal exopolyphosphatase from *Leishmania major* with high affinity for short chain polyphosphate. J Biol Chem 277(52):50899–50906. doi:10.1074/jbc.M208940200

Rodrigues CO, Ruiz FA, Rohloff P et al (2002b) Characterization of isolated acidocalcisomes from *Toxoplasma gondii* tachyzoites reveals a novel pool of hydrolyzable polyphosphate. J Biol Chem 277(50):48650–48656. doi:10.1074/jbc.M208990200

Rohloff P, Rodrigues CO, Docampo R (2003) Regulatory volume decrease in *Trypanosoma cruzi* involves amino acid efflux and changes in intracellular calcium. Mol Biochem Parasitol 126(2):219–230

Rohloff P, Montalvetti A, Docampo R (2004) Acidocalcisomes and the contractile vacuole complex are involved in osmoregulation in *Trypanosoma cruzi*. J Biol Chem 279(50):52270–52281. doi:10.1074/jbc.M410372200

Rohloff P, Miranda K, Rodrigues JC et al (2011) Calcium uptake and proton transport by acidocalcisomes of *Toxoplasma gondii*. PLoS One 6(4):e18390. doi:10.1371/journal.pone.0018390

Rooney PJ, Ayong L, Tobin CM et al (2011) TgVTC2 is involved in polyphosphate accumulation in *Toxoplasma gondii*. Mol Biochem Parasitol 176(2):121–126. doi:10.1016/j.molbiopara.2010.12.012

Ruiz FA, Rodrigues CO, Docampo R (2001a) Rapid changes in polyphosphate content within acidocalcisomes in response to cell growth, differentiation, and environmental stress in *Trypanosoma cruzi*. J Biol Chem 276(28):26114–26121. doi:10.1074/jbc.M102402200

Ruiz FA, Marchesini N, Seufferheld M et al (2001b) The polyphosphate bodies of *Chlamydomonas reinhardtii* possess a proton-pumping pyrophosphatase and are similar to acidocalcisomes. J Biol Chem 276(49):46196–46203. doi:10.1074/jbc.M105268200

Ruiz FA, Lea CR, Oldfield E, Docampo R (2004a) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem J Biol Chem 279(43):44250–44257. doi:10.1074/jbc.M406261200

Ruiz FA, Luo S, Moreno SN, Docampo R (2004b) Polyphosphate content and fine structure of acidocalcisomes of *Plasmodium falciparum*. Microsc Microanal 10(5):563–567. doi:10.1017/S1431927604040875

Sahin A, Espiau B, Tetaud E et al (2008) The leishmania ARL-1 and Golgi traffic. PLoS One 3(2):e1620. doi:10.1371/journal.pone.0001620

Scott DA, Docampo R (2000) Characterization of isolated acidocalcisomes of *Trypanosoma cruzi*. J Biol Chem 275(31):24215–24221. doi:10.1074/jbc.M002454200

Scott DA, Docampo R, Dvorak JA et al (1997) In situ compositional analysis of acidocalcisomes in *Trypanosoma cruzi*. J Biol Chem 272(44):28020–28029

Sethuraman A, Rao NN, Kornberg A (2001) The endopolyphosphatase gene: essential in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 98(15):8542–8547. doi:10.1073/pnas.151269398

Seufferheld M, Vieira MC, Ruiz FA et al (2003) Identification of organelles in bacteria similar to acidocalcisomes of unicellular eukaryotes. J Biol Chem 278(32):29971–29978. doi:10.1074/jbc.M304548200

Seufferheld M, Lea CR, Vieira M et al (2004) The H^+-pyrophosphatase of *Rhodospirillum rubrum* is predominantly located in polyphosphate-rich acidocalcisomes. J Biol Chem 279(49):51193–51202. doi:10.1074/jbc.M406099200

Shi X, Kornberg A (2005) Endopolyphosphatase in *Saccharomyces cerevisiae* undergoes post-translational activations to produce short-chain polyphosphates. FEBS Lett 579(9):2014–2018. doi:10.1016/j.febslet.2005.02.032

Smith SA, Mutch NJ, Baskar D et al (2006) Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci U S A 103(4):903–908. doi:10.1073/pnas.0507195103

Soares Medeiros LC, Gomes F, Maciel LR et al (2011) Volutin granules of *Eimeria* parasites are acidic compartments and have physiological and structural characteristics similar to acidocalcisomes. J Eukaryot Microbiol 58(5):416–423. doi:10.1111/j.1550-7408.2011.00565.x

Ulrich PN, Lander N, Kurup SP et al (2014) The acidocalcisome vacuolar transporter chaperone 4 catalyzes the synthesis of polyphosphate in insect-stages of *Trypanosoma brucei* and *T. cruzi*. J Eukaryot Microbiol 61(2):155–165. doi:10.1111/jeu.12093

Vercesi AE, Moreno SN, Docampo R (1994) Ca^{2+}/H^+ exchange in acidic vacuoles of *Trypanosoma brucei*. Biochem J 304(Pt 1):227–233

Whitehead MP, Hooley P, Brown MRW (2013) Horizontal transfer of bacterial polyphosphate kinases to eukaryotes: implications for the ice age and land colonisation. BMC Res Notes 6:221. doi:10.1186/1756-0500-6-221

Yagisawa F, Nishida K, Yoshida M et al (2009) Identification of novel proteins in isolated polyphosphate vacuoles in the primitive red alga *Cyanidioschyzon merolae*. Plant J: Cell Mol Biol 60(5):882–893. doi:10.1111/j.1365-313X.2009.04008.x

Zhang K, Hsu FF, Scott DA et al (2005) *Leishmania* salvage and remodelling of host sphingolipids in amastigote survival and acidocalcisome biogenesis. Mol Microbiol 55(5):1566–1578. doi:10.1111/j.1365-2958.2005.04493.x

Zhang H, Gomez-Garcia MR, Shi X et al (2007) Polyphosphate kinase 1, a conserved bacterial enzyme, in a eukaryote, *Dictyostelium discoideum*, with a role in cytokinesis. Proc Natl Acad Sci U S A 104(42):16486–16491. doi:10.1073/pnas.0706847104

Inorganic Polyphosphates in Mycorrhiza

4

Tatsuhiro Ezawa, Chiharu Tani, Nowaki Hijikata, and Yusuke Kikuchi

Abstract

Mycorrhizas are the mutualistic associations between fungi and plant roots. Mycorrhizal fungi construct hyphal networks both in the soil (extraradical mycelia) and root cortex (intraradical mycelia), take up mineral nutrients, in particular phosphate, from the soil, and deliver to the host. In turn, the host supplies carbon source to the fungi. Phosphate is an essential nutrient for plants, but its diffusion in the soil is slow. Extraradical mycelia can explore a larger volume of soil than roots for phosphate acquisition, and the fungi deliver phosphate to the host more rapidly than the diffusion of P_i in the soil. Similar to "polyphosphate overplus" in yeast and bacteria, mycorrhizal fungi are capable of accumulating a massive amount of polyphosphate in the vacuoles quite rapidly, which provides large buffering capacity and storage of phosphate that is to be delivered to the host. Phosphate is taken up via H^+/phosphate and Na^+/phosphate symporters, incorporated to γ-phosphate of ATP, polymerized by the vacuolar transporter chaperone complex in the tonoplast using ATP as a phosphoryl donor, and released into the vacuole, during which Na^+, K^+, Mg^{2+}, and Ca^{2+} are taken up via cation transporters synchronously and equivalently with polyphosphate accumulation to maintain charge neutrality of the cell. The genes encoding these transporters and ATPases are upregulated in response to increased external phosphate concentration, implying that polyphosphate accumulation is achieved by orchestrated regulation of the expression of various genes. Although the molecular mechanism of polyphosphate accumulation has recently been studied extensively, driving force for polyphosphate translocation toward the host of mycorrhizal fungi has not been well understood.

T. Ezawa (✉) • C. Tani • N. Hijikata • Y. Kikuchi
Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
e-mail: tatsu@res.agr.hokudai.ac.jp

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_4

4.1 Mycorrhizal Symbiosis

Mycorrhizas are the mutualistic associations between fungi and plant roots. Arbuscular mycorrhizal (AM) fungi that belong to the ancient phylum Glomeromycota are most widespread, i.e., associated with 70–90 % of land plants (Smith and Read 2008). AM associations occurred more than 400 million years ago; the fossil records (Remy et al. 1994) and estimation by molecular evolutionary clock (Simon et al. 1993) indicate AM fungi coincided with the appearance of early land plants, which suggests that AM symbiosis was instrumental in the colonization of primitive plants toward land. During the evolution and diversification of land plants, however, distinct mycorrhizal associations evolved; ectomycorrhizas are associations between woody species and fungi, in the Basidiomycota and Ascomycota (Tedersoo et al. 2010), whereas ericaceous plants form ericoid mycorrhizas mainly with ascomycotan fungi, but also with some basidiomycotan fungi (Straker 1996). Host-fungal specificity is strict in some ectomycorrhizal and ericoid mycorrhizal symbioses (Tedersoo et al. 2010; Villarreal-Ruiz et al. 2004; Straker 1996), while AM fungi generally show no apparent host specificity, at least in glasshouse experiments (Smith and Read 2008). Despite the phylogenetic distances among these mycorrhizal fungi, the associations are generally mutualistic. The fungal partners take up mineral nutrients, in particular phosphorus (P), from the soil and deliver to the host plant, and in turn, the host supplies carbon source to the fungi (Smith and Read 2008).

Inorganic orthophosphate (P_i) is an essential nutrient for plants, but poorly mobile (diffusive) in the soil, because P_i forms sparingly soluble salts with aluminum and iron that are abundant in the soil. Therefore, plants have developed various strategies for improving P_i uptake efficiency, e.g., the formation of root hair/lateral roots to extend surface area for P_i uptake and the exudation of organic acids to chelate the counter ions of sparingly soluble P_i salts (Lambers et al. 2008). Mycorrhizal associations are a distinct strategy for P_i acquisition in plants. The fungi take up P_i from soil solution through extensive hyphal networks constructed in the soil (i.e., extraradical mycelia) far from the root surface and translocate P_i to the root-fungal interface; the translocation rate is more rapid than the diffusion rate of P_i in the soil solution (Smith et al. 2011). Then the P_i is released from the fungus to the host at the interface that is distinctively different among the three mycorrhizas. AM fungi form highly branched hyphal terminus “arbuscules” on intraradical mycelia in the root cortex (Fig. 4.1). Hartig net is a hyphal network formed between the root epidermal cells in ectomycorrhizas, while ericoid mycorrhizal fungi form hyphal coils in the epidermal cells (detailed structures are described by Mark Brundrett at http://mycorrhizas.info/index.html). In addition, the individual fungal hyphae have much smaller diameters than roots and thus are able to access to narrower soil pores, which allow the fungi exploring more soil volume (Drew et al. 2003). Therefore, mycorrhizal fungi have been playing a significant role in the establishment of terrestrial vegetation via enhancing P cycling.

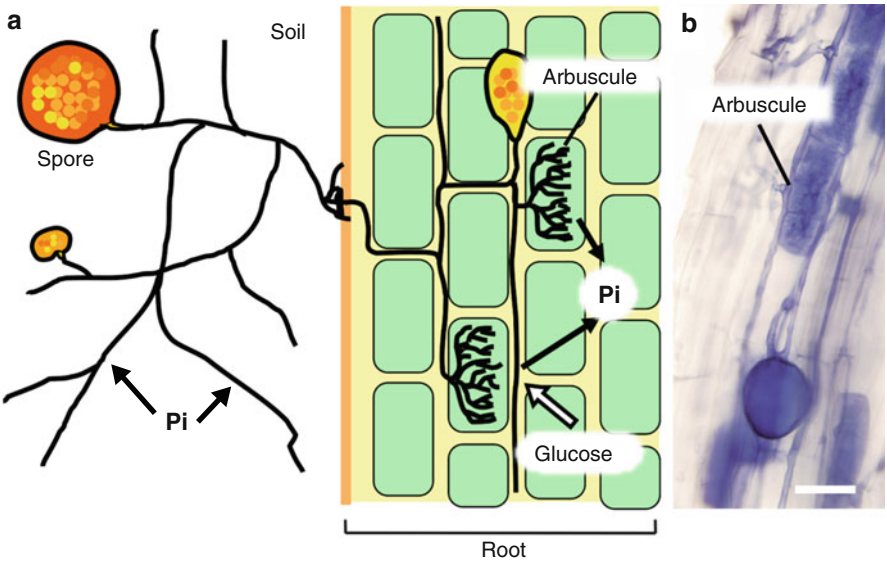

Fig. 4.1 Arbuscular mycorrhiza. (**a**) Spore of arbuscular mycorrhizal fungi in the soil germinates, colonizes root cortex, and constructs hyphal networks both in the soil (extraradical mycelium) and root cortex (intraradical mycelium). Phosphate (P_i) is taken up from the soil by extraradical hyphae, translocated through hyphae to arbuscules, and released to the periarbuscular space (apoplast between the root and fungal cells). In turn, the host plant supplies glucose to the fungi. (**b**) Typical structure of intraradical mycelium of arbuscular mycorrhizal fungi. Soybean roots collected from the field were stained with trypan blue and observed with an optical microscope (Bar, 10 μm)

4.2 Identification of Polyphosphate in Mycorrhizal Fungi

It is surprising that the inorganic polyphosphate (polyP) fraction in mycorrhizal fungi had already been recognized in the early 1960s as "bound-form phosphate" that appeared in the mycorrhizal roots immediately after exposure to P_i solution and comprised a much larger phosphate pool than orthophosphate in the fungal tissue (Jennings 1964; Harley and Loughman 1963). Classical studies on the ectomycorrhizas of beech demonstrated that in the presence of the intermediate concentrations of P_i (50–1,000 μM), a large proportion of P_i taken up by the fungi was incorporated into the bound-form phosphate (i.e., large phosphate pool) that was slowly remobilized and transferred to the host when the external P_i concentration fell below 50 μM (Jennings 1964; Harley and Loughman 1963; Harley et al. 1956). In the 1970s, Ashford and her colleagues hypothesized that the bound-form phosphate is polyP and conducted various cytochemical tests using the ectomycorrhizas of *Eucalyptus* sp.; they demonstrated that the metachromatic granules appeared during P_i uptake were polyP that corresponds to the bound-form phosphate (Ashford et al. 1975). Thereafter, polyP was extracted from onion roots colonized with the AM fungus *Glomus mosseae*, separated from RNA and DNA by gel electrophoresis, and

quantified after acid digestion; polyP represented a substantial proportion of the total fungal P, strongly suggesting that polyP is the fraction that is translocated to the host (Callow et al. 1978). After these pioneer studies, polyP was studied mostly in ectomycorrhizal and ericoid mycorrhizal fungi (e.g., Straker and Mitchell 1985; Martin et al. 1985), but not much in AM fungi. This might be because many of ectomycorrhizal and ericoid mycorrhizal fungi are culturable *in vitro*, but AM fungi are not (i.e., obligate biotrophs that proliferate only in the presence of host plant).

In these early studies, polyP was considered to be present as granules *in vivo*, but it was also Ashford's group who questioned the granular polyP. During the 1970s–1990s, polyP was generally identified by metachromay (toluidine blue O staining) or by yellow fluorescence (DAPI staining) after chemical fixation. Orlovich and Ashford (1993) found that the metachromatic granules were absent after glutaraldehyde fixation, but appeared during the ethanol dehydration processes in the ectomycorrhizal fungus *Pisolithus tinctorius*. Moreover, X-ray microanalysis showed that in freeze-substituted hyphae (in which diffusion and precipitation of elements could be minimized), P was not present as a granular form in the vacuoles, but evenly distributed throughout the vacuoles, suggesting that the majority of polyP is present as a soluble form *in vivo*, and thus the granules are artifacts. This idea was further supported by the light microscopic observations that demonstrated the absence of the granule-like material in vacuoles of living hyphae (Ashford et al. 1999; Hyde and Ashford 1997). In contrast, Bücking and her colleagues demonstrated granular polyphosphate in the vacuoles of living and freeze-substituted hyphae of *P. tinctorius* (Bücking and Heyser 1999; Bücking et al. 1998). Granular (precipitated) polyP would be metabolically inert and relatively immobile, whereas soluble polyP is more mobile and thus could readily be translocated to the host. Therefore, the status of polyP in the cell was an important issue to consider the physiological role of polyP in mycorrhiza. This issue is further discussed in the following section.

4.3 Technical Breakthroughs for Specific Detection of Polyphosphate

For specific detection of polyP at the subcellular level, polyP-binding domain (PPBD) of *Escherichia coli* exopolyphosphatase was trimmed, tagged with an epitope, and used to localize polyP in *Saccharomyces cerevisiae* (Saito et al. 2005); even in the yeast grown at 10 mM P_i, under which the yeast accumulated 340-fold more polyP than that grown at 0.2 mM P_i, polyP was not present as granules but evenly distributed in the vacuoles. Using this technique, uniform (dispersed) distribution of polyP in vacuoles was also demonstrated in the dark septate root endophyte *Phialocephala fortinii* (Saito et al. 2006). Vacuolar polyP status was also carefully examined using rapidly frozen and freeze-substituted germ tubes of the AM fungus *Gigaspora margarita* (Kuga et al. 2008). DAPI staining showed uniform distribution of polyP in the vacuoles (Fig. 4.2), which was supported both by affinity labeling with the PPBD and by P mapping by energy-filtering transmission electron

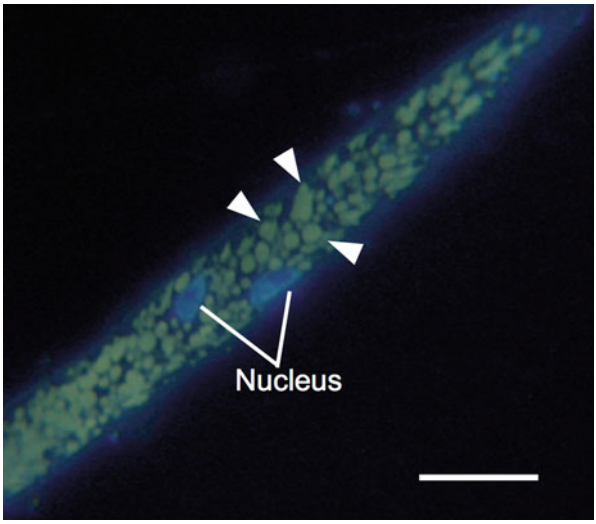

Fig. 4.2 Polyphosphate status in the vacuoles of *Gigaspora margarita* germ tube. The germ tube was rapidly frozen, freeze substituted, embedded in resin, sectioned, stained with DAPI, and observed by a scanning confocal microscope. Numerous vacuoles that are uniformly stained by DAPI are present in the germ tube (*arrowheads*), suggesting that polyphosphate is present as a soluble form, but not as granules (Reproduced by courtesy of Katsuharu Saito)

microscopy. It is likely that host sink activity affects cellular polyP status of the fungal symbionts; if P demand of host plant is high, most polyP would be solubilized and dynamically turning over to deliver to the host rapidly. To test this idea, not only microscopic observations but also quantitative analysis was necessary.

A highly sensitive and specific method for polyP quantification was developed in Kornberg's laboratory using another *E. coli* enzyme polyP kinase (PPK) in conjunction with luciferase-catalyzed luminescence (Ault-Riché et al. 1998). In this method, PPK transfers the terminal P_i residue of polyP to ADP (i.e., PPK reverse reaction), and the resultant ATP is measured by the luciferin/luciferase system. PPK showed higher affinity to polyPs longer than 23 P_i residues, and processing of polyPs with ten P_i residues or shorter was limited (Ohtomo et al. 2004). On the other hand, *S. cerevisiae* exopolyphosphatase (PPX) was found to react more uniformly with short-chain polyPs (Ohtomo et al. 2008). The detection limit of the PPK method is, however, more than an order of magnitude lower than that of the PPX method. Furthermore, given that the dynamic range of the PPK method is much wider than that of the PPX method, the PPK method seems to be applicable for a smaller amount of sample and a wider range of biological material. The establishment of PPK method provided a significant breakthrough for understanding of the dynamics and metabolism of polyP in mycorrhizal fungi.

4.4 Dynamics of Polyphosphate in Mycorrhizal Fungi

PolyP dynamics in AM fungi has extensively been investigated by applying the PPK method. Extraradical mycelia (i.e., hyphal networks in the soil) are the primary site responsible for P_i uptake and polyP accumulation. Under P-deficient conditions, AM fungi are capable of accumulating a massive amount of polyP quite rapidly,

similar to "polyP overplus" described in yeast and bacteria in classical studies (reviewed in Harold 1966). An AM fungus *Archaeospora leptoticha* accumulated polyP at a rate of 46 nmol P_i min^{-1} mg^{-1} protein in response to application of high concentration (1 mM) of Pi, comparable to the rate observed in polyP-hyperaccumulating bacterium *Acinetobacter johnsonii* (98 nmol P_i min^{-1} mg^{-1} protein) isolated from sewage sludge (Ezawa et al. 2004). The maximum amount of polyP in extraradical mycelia was 7 μmol P_i mg^{-1} protein (corresponding to 400–500 μmol g^{-1} DW) and reached to 64 % of the total P in *Rhizophagus clarus* (Hijikata et al. 2010). It is noteworthy that during the polyP overplus, no fluctuation in cellular P_i level was observed. This indicates that polyP synthesis is rapid enough to maintain cytosolic P_i concentration within an optimal range, which enables rapid and continuous P_i uptake without disrupting cellular homeostasis. All these results suggest that polyP provides the largest P storage that is flexibly operated in response to external P_i concentration.

The polyP accumulated in extraradical mycelia was translocated to intraradical mycelia (mycelia in the root) within 10–12 h after P_i application (Kikuchi et al. 2014; Hijikata et al. 2010). PolyP is likely to be hydrolyzed to P_i in arbuscules and released into the interfacial apoplast between the arbuscule and root cortical cell (Ezawa et al. 2002). Differentiation in polyP metabolism between extraradical and intraradical mycelia has been extensively studied. Chain-length distribution of polyP is different between extraradical and intraradical mycelia. Successive extractions with trichloroacetic acid, EDTA, and phenol-chloroform that solubilize short-chain, long-chain, and granular polyPs revealed that chain length was shorter in intraradical mycelia (Solaiman et al. 1999). PolyP in intraradical and extraradical mycelia was extracted, fractionated by gel-filtration spin columns with different molecular weight cutoff (2 or 6 kDa), and quantified by the PPK method; low-molecular weight (i.e., short chain length) polyPs were more abundant in the intraradical mycelia (Ohtomo et al. 2004). Takanishi et al. (2009) employed both the PPK and PPX methods and found that PPX- detectable, but PPK- undetectable polyPs, i.e., polyPs of 20 or less P_i residues occurred more in intraradical mycelia. This difference in polyP chain length was reflected in difference in polyP-hydrolyzing activity; the activity in intraradical mycelia showed lower K_m for short-chain polyP than for long-chain polyP, whereas that in extraradical mycelia showed lower K_m for long-chain polyP (Ezawa et al. 2001). These results suggest that polyP may not be an inert fraction but dynamically turning over during translocation, which is further discussed in the next section.

4.5 Cellular Processes of Polyphosphate Accumulation

Fungi accumulate a vast amount of polyP in the cell, e.g., yeast accumulates up to 10 % of dry weight (Kornberg et al. 1999), but the biosynthetic pathway for polyP was a long-lasting mystery in eukaryotes. Although several polyP-defective yeast mutants were isolated (e.g., Ogawa et al. 2000), enzyme(s)/gene(s) that are directly involved in the biosynthesis had not been identified. In fungi, there is no doubt that polyP is accumulated in the vacuoles (Kuga et al. 2008; Saito et al. 2005, 2006;

Viereck et al. 2004; Kornberg et al. 1999; Ashford et al. 1999). In addition, the fact that polyP is a linear chain of P_i residues linked by high-energy phosphoanhydride bonds suggests that the polymer is synthesized using the γ-P_i of ATP as a phosphoryl donor. Therefore, it was hypothesized that polyP-synthesizing enzyme is localized in the tonoplast, polymerizes P_i using ATP supplied from the cytosol, and releases polyP into the vacuole. This hypothesis was clearly certified in *S. cerevisiae* by Hothorn et al. (2009). The vacuolar transporter chaperone (VTC) complex that is a hetero-oligomer of VTC proteins was found to be responsible for polyP biosynthesis, and among the proteins, the catalytic core was present in that encoded by *VTC4*. We could also successfully demonstrate polyP-synthesizing activity in the tonoplast fraction of the AM fungus *R. clarus*, although this was the second case in eukaryotes (Tani et al. 2009).

PolyP is polyanionic compound that has one negative charge per P_i residue in addition to the two negative charges of the terminal residues. This implies that the accumulation of polyP in the cell results in accumulation of negative charge that should be neutralized to maintain cellular homeostasis. Until recently, however, little was known about how charge neutrality is maintained during polyP overplus. We hypothesized that AM fungi take up inorganic cations or accumulate basic (cationic) amino acid, such as arginine that is abundant in the fungi (Govindarajulu et al. 2005), to neutralize the negative charge. *R. clarus* was grown in association with the host plant *Lotus japonicus* or *Nicotiana benthamiana* under P-deficient conditions, and polyP accumulation in extraradical mycelia was triggered by applying 1 mM P_i solution. With increasing polyP, four inorganic cations, Na^+, K^+, Ca^{2+}, and Mg^{2+}, were synchronously taken up by the mycelia (Kikuchi et al. 2014), consistent with the microscopic observation with X-ray microanalysis in which these four cations were colocalized in the vacuoles of *R. irregularis* (Bücking and Shachar-Hill 2005). The total electric charge of these four cations, moreover, was increased and decreased equivalently to the total negative charge of polyP. On the other hand, no drastic increase/decrease in the levels of amino acids, including arginine, during polyP accumulation was observed. To understand the molecular mechanism underlying the polyP accumulation, transcriptome analysis was conducted on the Illumina platform (Kikuchi et al. 2014). The application of 1 mM P_i to the P-starved mycelia induced expression of *PHO84* (H^+/P_i symporter), *PHO89* (Na^+/P_i symporter), and genes encoding H^+- and Na^+-ATPases that drive these symporters (Fig. 4.3). In addition, various cation transporter genes responsible for cation uptake across plasma membrane and tonoplast were also upregulated, which strengthened the finding that the cations are taken up synchronously and equivalently with polyP accumulation. It was intriguing that not only *VTC*s (polyP polymerase) but also *PPN* (endopolyphosphatase) and *PHO91* (vacuolar P_i exporter) are upregulated in response to P_i application, supporting the idea that polyP is dynamically turning over during translocation. It was predicted that energy generation pathways would be activated because a large amount of ATP would be consumed for P_i/cation uptake and polyP synthesis. Unexpectedly, however, most genes involved in the major pathways, e.g., glycolysis, TCA cycle, electron transfer chain, and β-oxidation, were unresponsive to P_i application. In fact, ATP levels did not fluctuate during

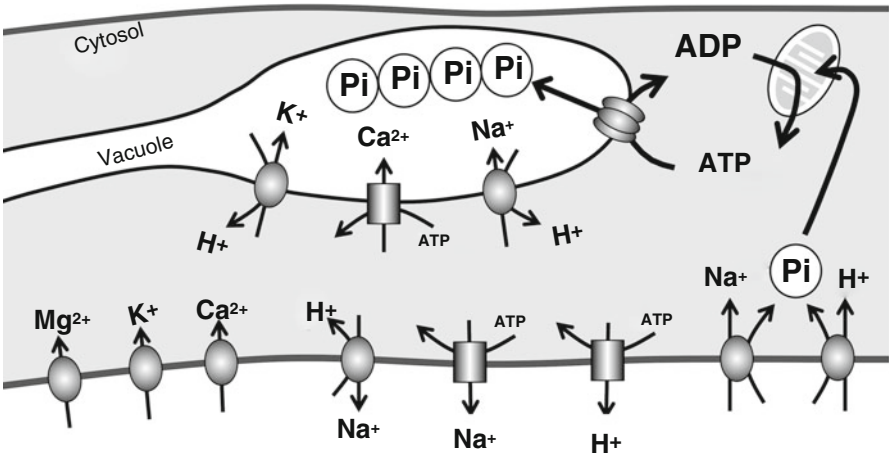

Fig. 4.3 Schematic representation of cellular processes of polyphosphate accumulation in arbuscular mycorrhizal fungi. Phosphate (P_i) is taken up via H^+/P_i and Na^+/P_i symporters that are driven by H^+- and Na^+-ATPases, respectively, incorporated to γ-P_i of ATP through oxidative phosphorylation in mitochondria, polymerized into polyphosphate by VTC complex on the tonoplast, and accumulated in the vacuole. Na^+, K^+, Mg^{2+}, and Ca^{2+} are taken up via plasma membrane-type cation transporters and sequestered into the vacuole via vacuolar-type cation transporters synchronously and equivalently with polyphosphate accumulation

polyP accumulation. Overall, the study demonstrated that polyP overplus is concurrent with the rapid uptake of the inorganic cations, which is achieved by the orchestrated regulation of gene expression.

4.6 Perspectives

The discovery of motile tubular vacuoles has a significant impact on understanding long-distance nutrient transport in filamentous fungi (Shepherd et al. 1993b). In particular, given that polyP is accumulated in vacuoles (Kuga et al. 2008; Saito et al. 2006; Viereck et al. 2004; Ashford et al. 1994), the motility and continuity of vacuolar system are implicated in the efficiency of P delivery in mycorrhizas. In earlier studies, protoplasmic streaming, e.g., motor protein-mediated vesicle transport, were considered to play a major role in nutrient translocation in fungi. Ashford and colleagues extensively studied on the vacuolar system using the ectomycorrhizal fungus *P. tinctorius* as a model (e.g., Cole et al. 1997, 1998; Shepherd et al. 1993a) and provided a novel perspective for solute transport in filamentous fungi; the major nutrients, including P_i and polyP, are translocated through the vacuolar system (Ashford and Allaway 2002), to which a simple (bidirectional) diffusion model along source-sink gradients fitted (Darrah et al. 2006). Obviously, only "soluble polyP", but not "granular polyP", could be translocated through this system. In AM fungi motile tubular vacuoles were also demonstrated (Uetake et al. 2002), and the

involvement of the vacuoles in long-distance polyP translocation is highly likely (Kuga et al. 2008). The idea that (nondirectional) simple diffusion is the only driving force for polyP translocation, however, is questioned, particularly in mycorrhizal associations, due to the following reasons. Firstly, the simple diffusion model by Darrah et al. (2006) was constructed based on the observations on *in vitro* pure culture without associating with a plant, but rates of polyP translocation seem more rapid in the direction toward the host when the fungal symbiont is associated with an autotrophic plant (e.g., Hijikata et al. 2010). Secondly, a classical study by (Cooper and Tinker 1981) demonstrated that the inhibition of transpiration of the host plant suppressed P delivery from the fungus. Although the authors considered that the inhibition of transpiration suppressed photosynthesis and thus P delivery via reducing energy supply to the fungus, these observations led us to hypothesize that there is mass flow (probably driven by transpiration) through hyphae that may provide a driving force for polyP translocation. This idea is supported by the observation that several water channel (aquaporin) genes are expressed both in intra- and extraradical hyphae, suggesting that there is mass flow from extraradical hyphae to intraradical hyphae (Kikuchi and Ezawa, unpublished observations). In fact, there is increasing evidence that mycorrhizal fungi transport water to the host both in ectomycorrhizal and AM associations (Li et al. 2013; Xu et al. 2015). These questions remain to be clarified in the future.

PolyP in mycorrhizal fungi provides large buffering capacity and storage of P_i that is to be delivered to the host plants and thus has important implications in the long history of coevolution between plants and mycorrhizal fungi. On the other hand, the fact that genes directly responsible for polyP accumulation and turnover, e.g., *VTC*s, *PHO91*, and *PPN*, are widely conserved among phylogenetically distant fungi suggests that these genes are essential for survival not only in mycorrhizal fungi but also in other fungi. It has been well documented that polyP is involved in the expression of virulence in many of human pathogenic bacteria (Rao et al. 2009) and protists (Moreno and Docampo 2013), but so far little is known about whether polyP is essential for virulence expression of pathogenic fungi, in particular plant pathogenic fungi. Fungi that interact with plants are categorized into two extreme groups with respect to life-history strategy; one consists of symbiotic mycorrhizal fungi, and the other is of pathogenic (parasitic) fungi. In the former group, the significance of polyP in the interactions has already been discussed in this chapter, whereas that in the latter group is also of interest. For example, the polyP polymerase gene *VTC4* is widely conserved at least in the genomes of the following major plant pathogens, *Magnaporthe oryzae*, *Fusarium verticillioides*, *Verticillium dahliae*, *Rosellinia necatrix*, *Sclerotinia sclerotiorum*, and *Ophiostoma piceae*, leading to the idea that polyP also play a significant role in plant-pathogen interactions. A preliminary experiment is currently being undertaken in collaboration with a plant pathologist group.

Acknowledgments The studies on polyP dynamics and comparative transcriptome analysis in AM fungi were partially supported by the Grant-in-Aid for Scientific Research (17380043, 19380040, and 22380042) from the Japan Society for the Promotion of Science (TE) and conducted

in the open research facility in Hokkaido Agricultural Research Center, National Agricultural Research Organization (NH, CT, YK, and TE). The PPK was kindly supplied by Dr. R. Ohtomo. The authors also thank Drs. M. Saito, K. Saito, Y. Kuga, Y. Handa, and M. Kawaguchi for their invaluable comments and suggestions.

References

Ashford AE, Allaway WG (2002) The role of the motile tubular vacuole system in mycorrhizal fungi. Plant Soil 244(1):177–187. doi:10.1023/a:1020271121683

Ashford AE, Lee ML, Chilvers GA (1975) Polyphosphate in eucalypt mycorrhizas: a cytochemical demonstration. New Phytol 74:447–453

Ashford AE, Ryde S, Barrow KD (1994) Demonstration of a short chain polyphosphate in *Pisolithus tinctorius* and the implications for phosphorus transport. New Phytol 126(2):239–247. doi:10.1111/j.1469-8137.1994.tb03942.x

Ashford AE, Vesk PA, Orlovich DA et al (1999) Dispersed polyphosphate in fungal vacuoles in *Eucalyptus pilularis*/*Pisolithus tinctorius* ectomycorrhizas. Fungal Genet Biol 28(1):21–33, http://dx.doi.org/10.1006/fgbi.1999.1140

Ault-Riché D, Fraley CD, Tzeng CM et al (1998) Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. J Bacteriol 180(7):1841–1847

Bücking H, Heyser W (1999) Elemental composition and function of polyphosphates in ectomycorrhizal fungi – an X-ray microanalytical study. Mycol Res 103(1):31–39

Bücking H, Shachar-Hill Y (2005) Phosphate uptake, transport and transfer by the arbuscular mycorrhizal fungus *Glomus intraradices* is stimulated by increased carbohydrate availability. New Phytol 165(3):899–912

Bücking H, Beckmann S, Heyser W et al (1998) Elemental contents in vacuolar granules of ectomycorrhizal fungi measured by EELS and EDXS. A comparison of different methods and preparation techniques. Micron 29(1):53–61, http://dx.doi.org/10.1016/S0968-4328(97)00059-0

Callow JA, Capaccio LCM, Parish G et al (1978) Detection and estimation of polyphosphate in vesicular-arbuscular mycorrhizas. New Phytol 80:125–134

Cole L, Hyde GJ, Ashford AE (1997) Uptake and compartmentalisation of fluorescent probes by Pisolithus tinctorius hyphae: evidence for an anion transport mechanism at the tonoplast but not for fluid-phase endocytosis. Protoplasma 199:18–29

Cole L, Orlovich DA, Ashford AE (1998) Structure, function, and motility of vacuoles in filamentous fungi. Fungal Genet Biol 24(1–2):86–100

Cooper KM, Tinker PB (1981) Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. IV. Effect of environmental variables on movement of phosphorus. New Phytol 88:327–339

Darrah PR, Tlalka M, Ashford A et al (2006) The vacuole system is a significant intracellular pathway for longitudinal solute transport in basidiomycete fungi. Eukaryot Cell 5(7):1111–1125. doi:10.1128/ec.00026-06

Drew EA, Murray RS, Smith SE, Jakobsen I et al (2003) Beyond the rhizosphere: growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. Plant Soil 251(1):105–114. doi:10.1023/A:1022932414788

Ezawa T, Smith SE, Smith FA (2001) Differentiation of polyphosphate metabolism between the extra- and intraradical hyphae of arbuscular mycorrhizal fungi. New Phytol 149(3):555–563

Ezawa T, Smith S, Smith FA (2002) P metabolism and transport in AM fungi. Plant Soil 244(1–2):221–230. doi:10.1023/A:1020258325010

Ezawa T, Cavagnaro TR, Smith SE et al (2004) Rapid accumulation of polyphosphate in extraradical hyphae of an arbuscular mycorrhizal fungus as revealed by histochemistry and a polyphosphate kinase/luciferase system. New Phytol 161(2):387–392. doi:10.1046/j.1469-8137.2003.00966.x

Govindarajulu M, Pfeffer PE, Jin HR et al (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435(7043):819–823

Harley JL, Loughman BC (1963) The uptake of phosphate by excised mycorrhizal roots of the beech. New Phytol 62(3):350–359. doi:10.1111/j.1469-8137.1963.tb06340.x

Harley JL, McCready CC, Brierley JK et al (1956) The salt respiration of excised beech mycorrhizas II. The relationship between oxygen consumption and phosphate absorption. New Phytol 55(1):1–28

Harold FM (1966) Inorganic polyphosphates in biology: structure, metabolism, and function. Bacteriol Rev 30(4):772–794

Hijikata N, Murase M, Tani C et al (2010) Polyphosphate has a central role in the rapid and massive accumulation of phosphorus in extraradical mycelium of an arbuscular mycorrhizal fungus. New Phytol 186(2):285–289. doi:10.1111/j.1469-8137.2009.03168.x

Hothorn M, Neumann H, Lenherr ED et al (2009) Catalytic core of a membrane-associated eukaryotic polyphosphate polymerase. Science 324(5926):513–516. doi:10.1126/science.1168120

Hyde GJ, Ashford AE (1997) Vacuole motility and tubule-forming activity in *Pisolithus tinctorius* hyphae are modified by environmental conditions. Protoplasma 198(1–2):85–92

Jennings DH (1964) Changes in the size of orthophosphate pools in mycorrhizal roots of beech with reference to absorption of the ion from the external medium. New Phytol 63(2):181–193. doi:10.1111/j.1469-8137.1964.tb07370.x

Kikuchi Y, Hijikata N, Yokoyama K et al (2014) Polyphosphate accumulation is driven by transcriptome alterations that lead to near-synchronous and near-equivalent uptake of inorganic cations in an arbuscular mycorrhizal fungus. New Phytol 204(3):638–649. doi:10.1111/nph.12937

Kornberg A, Rao NN, Ault-Riche D (1999) Inorganic polyphosphate: a molecule of many functions. Ann Rev Biochem 68(1):89–125. doi:10.1146/annurev.biochem.68.1.89

Kuga Y, Saito K, Nayuki K et al (2008) Ultrastructure of rapidly frozen and freeze-substituted germ tubes of an arbuscular mycorrhizal fungus and localization of polyphosphate. New Phytol 178(1):189–200

Lambers H, Raven JA, Shaver GR et al (2008) Plant nutrient-acquisition strategies change with soil age. Trends Ecol Evol 23(2):95–103

Li T, Hu Y-J, Hao Z-P et al (2013) First cloning and characterization of two functional aquaporin genes from an arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol 197(2):617–630. doi:10.1111/nph.12011

Martin F, Marchal JP, Timinska A et al (1985) The metabolism and physical state of polyphosphates in ectomycorrhizal fungi. A ^{31}P nuclear magnetic resonance study. New Phytol 101(2):275–290. doi:10.1111/j.1469-8137.1985.tb02835.x

Moreno SNJ, Docampo R (2013) Polyphosphate and its diverse functions in host cells and pathogens. PLoS Pathog 9(5):e1003230. doi:10.1371/journal.ppat.1003230

Ogawa N, DeRisi J, Brown PO (2000) New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol Biol Cell 11(12):4309–4321

Ohtomo R, Sekiguchi Y, Mimura T et al (2004) Quantification of polyphosphate: different sensitivities to short-chain polyphosphate using enzymatic and colorimetric methods as revealed by ion chromatography. Anal Biochem 328(2):139–146. doi:10.1016/j.ab.2004.03.004

Ohtomo R, Sekiguchi Y, Kojima T et al (2008) Different chain length specificity among three polyphosphate quantification methods. Anal Biochem 383(2):210–216

Orlovich DA, Ashford AE (1993) Polyphosphate granules are an artefact of specimen preparation in the ectomycorrhizal fungus *Pisolithus tinctorius*. Protoplasma 173(3–4):91–102

Rao NN, Gómez-García MR, Kornberg A (2009) Inorganic polyphosphate: essential for growth and survival. Ann Rev Biochem 78(1):605–647. doi:10.1146/annurev.biochem.77.083007.093039

Remy W, Taylor TN, Hass H et al (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci U S A 91(25):11841–11843

Saito K, Ohtomo R, Kuga-Uetake Y et al (2005) Direct labeling of polyphosphate at the ultrastructural level in *Saccharomyces cerevisiae* by using the affinity of the polyphosphate binding domain of *Escherichia coli* exopolyphosphatase. Appl Environ Microbiol 71(10):5692–5701

Saito K, Kuga-Uetake Y, Saito M et al (2006) Vacuolar localization of phosphorus in hyphae of *Phialocephala fortinii*, a dark septate fungal root endophyte. Can J Microbiol 52:643–650

Shepherd VA, Orlovich DA, Ashford AE (1993a) Cell-to-cell transport via motile tubules in growing hyphae of a fungus. J Cell Sci 105(Part 4):1173–1178

Shepherd VA, Orlovich DA, Ashford AE (1993b) A dynamic continuum of pleiomorphic tubules and vacuoles in growing hyphae of a fungus. J Cell Sci 104(2):495–507

Simon L, Bousquet J, Levesque RC et al (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363(6424):67–69

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, London

Smith SE, Jakobsen I, Grønlund M et al (2011) Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant Physiol 156(3):1050–1057. doi:10.1104/pp.111.174581

Solaiman MZ, Ezawa T, Kojima T et al (1999) Polyphosphates in intraradical and extraradical hyphae of an arbuscular mycorrhizal fungus, *Gigaspora margarita*. Appl Environ Microbiol 65(12):5604–5606

Straker CJ (1996) Ericoid mycorrhiza: ecological and host specificity. Mycorrhiza 6(4):215–225. doi:10.1007/s005720050129

Straker CJ, Mitchell DT (1985) The characterization and estimation of polyphosphates in endomycorrhizas of the Ericaceae. New Phytol 99(3):431–440. doi:10.1111/j.1469-8137.1985.tb03670.x

Takanishi I, Ohtomo R, Hayatsu M et al (2009) Short-chain polyphosphate in arbuscular mycorrhizal roots colonized by *Glomus* spp.: a possible phosphate pool for host plants. Soil Biol Biochem 41(7):1571–1573, http://dx.doi.org/10.1016/j.soilbio.2009.04.002

Tani C, Ohtomo R, Osaki M et al (2009) Polyphosphate-synthesizing activity in extraradical hyphae of an arbuscular mycorrhizal fungus: ATP-dependent but proton gradient-independent synthesis. Appl Environ Microbiol 75(22):7044–7050. doi:10.1128/AEM.01519-09

Tedersoo L, May T, Smith M (2010) Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20(4):217–263. doi:10.1007/s00572-009-0274-x

Uetake Y, Kojima T, Ezawa T, Saito M (2002) Extensive tubular vacuole system in an arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 154:761–768. doi:10.1046/j.1469-8137.2002.00425.x

Viereck N, Hansen PE, Jakobsen I (2004) Phosphate pool dynamics in the arbuscular mycorrhizal fungus *Glomus intraradices* studied by *in vivo* ^{31}P NMR spectroscopy. New Phytol 162(3):783–794

Villarreal-Ruiz L, Anderson IC, Alexander IJ (2004) Interaction between an isolate from the *Hymenoscyphus ericae* aggregate and roots of *Pinus* and *Vaccinium*. New Phytol 164(1):183–192. doi:10.1111/j.1469-8137.2004.01167.x

Xu H, Kemppainen M, Kayal W et al (2015) Overexpression of *Laccaria bicolor* aquaporin JQ585595 alters root water transport properties in ectomycorrhizal white spruce (*Picea glauca*) seedlings. New Phytol 205(2):757–770. doi:10.1111/nph.13098

Functions of Inositol Polyphosphate and Inorganic Polyphosphate in Yeast and Amoeba

5

Adolfo Saiardi

Abstract

Phosphate, as a chemical group, forms a variety of bonds with important structural, informational and energetic roles. Therefore, the control of cellular phosphate homoeostasis is essential to cellular well-being. Key to this regulation of phosphate level is inorganic polyphosphate, a linear polymer of phosphate groups linked by phosphoanhydride bonds. While for many years bacterial research has dominated the inorganic polyphosphate field, interest in eukaryotic work is growing due to the discovery of this polymer's involvement in human diseases. Simple genetically tractable eukaryotes such as the yeast *Saccharomyces cerevisiae* and the social amoeba *Dictyostelium discoideum* have become excellent experimental models to study inorganic polyphosphate metabolism and physiological roles. The enzymes responsible for inorganic polyphosphate synthesis have been identified in both budding yeast and amoeba. In addition, research in yeast has revealed a strong metabolic connection between inorganic polyphosphate and inositol pyrophosphates, signalling molecules that belong to the vast and well-recognised inositol phosphates family. Interestingly, also inositol pyrophosphate metabolism and physiology has been primarily elucidated in the yeast and amoeba model organisms. The aim of the current essay is to highlight the metabolic and functional connections between these highly phosphorylated classes of molecules, focusing our attention on what the yeast and the amoeba have taught us about the functions and metabolism of inorganic polyphosphate and inositol pyrophosphates.

A. Saiardi
Medical Research Council Laboratory for Molecular Cell Biology (MRC-LMCB),
University College London, Gower Street, London WC1E 6BT, UK
e-mail: dmcbado@ucl.ac.uk

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_5

Abbreviations

IP_3	Inositol trisphosphate I(1,4,5)P_3
PP-IPs	Inositol pyrophosphates
polyP	Inorganic polyphosphate
PTM	Post-translational modification
PLC1	Phospholipase
IPMK	Inositol polyphosphate multikinase
PPK1	Inositol pentakisphosphate 2-kinase
IP6K	Inositol hexakisphosphate kinase
PPIP5K	Diphosphoinositol pentakisphosphate kinase
PPK	Polyphosphate kinase
IP_6	Inositol hexakisphosphate or phytic acid
VTC	Vacuolar transporter chaperone
WT	Wild type

5.1 Introduction

Phosphorus is one of the most common elements on earth. In cell it exists in its phosphate form (PO_4^{3-}) and plays fundamental and diverse roles. Phosphate performs a structural role in the charged backbone of nucleic acids as well as regulating cell behaviour through its ability to post-translationally modify proteins in signalling processes. Furthermore, phosphate groups linked together by the 'high-energy' phosphoanhydride bounds, as found in ATP, represent the principal biological energy store.

In eukaryote cells there are two groups of molecules especially rich in phosphate, inorganic polyphosphate (polyP) (Kornberg et al. 1999) and the inositol pyrophosphates (PP-IPs) (Saiardi 2012a). The polymer polyP contains from few to hundreds of phosphate residues linked by phosphoanhydride bonds (Fig. 5.1a). Although polyP is termed 'inorganic' – it contains no carbons – it is ubiquitously present in every living organism from bacteria to human. The cellular free phosphate concentration is buffered by polyP that due to its chelating property also regulates cation homoeostasis (Kornberg et al. 1999; Rao et al. 2009). Besides these basic attributes, polyP has chaperone-like activity (Gray et al. 2014) and regulates stress response (Jahid et al. 2006), ion channels (Zakharian et al. 2009; Seidlmayer et al. 2012) and infectivity (Moreno and Docampo 2013). Furthermore polyP appears to play a role in blood coagulation (Muller et al. 2009; Morrissey et al. 2012), though this function is debated (Faxalv et al. 2013).

Amongst the 'organic' (carbon containing) molecules, the PP-IPs are extraordinarily rich in phosphate. The best characterised inositol pyrophosphate, the diphosphoinositol pentakisphosphate (hereafter called IP_7), is notable for its arrangement of seven phosphates groups around the six-carbon inositol ring (Fig. 5.1b). Moreover, PP-IPs containing eight, nine or even more phosphates groups have been described (Losito et al. 2009; Pisani et al. 2014). Because several inositol pyrophosphates have the unique property of possessing more phosphates than carbon atoms, it is not unexpected that they too have been linked to phosphate homoeostasis. In

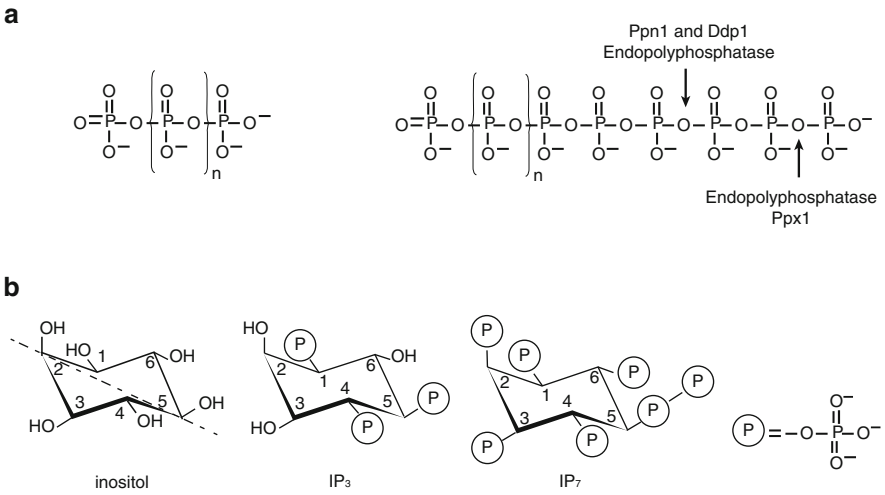

Fig. 5.1 Structure of inorganic polyphosphate and of typical inositol phosphates. Graphic representation of the minimal polyP structure (**a**, *left*) where n can range from 1 to several hundreds. Elongated representation of polyP molecule (**a**, *right*) showing the bonds targeted by the endopolyphosphatase activity of Ppn1 and Ddp1 and the exopolyphosphatase activity of Ppx1. Structure of myo-inositol (**b**, *left*); the *dashed line* between carbons 2 and 5 define the axis of symmetry on this molecule. Structure of the calcium-release factor IP_3 (**b**, *centre*) generated by the action of phospholipase C. The structure of the 'typical' IP_7 (**b**, *right*), the isomer 5PP-IP_5 (5-diphosphoinositol pentakisphosphate) generated by IP6Ks. *Circled P* indicates the phosphate group (PO_3^{2-})

fact the literature provides numerous examples of PP-IPs controlling cellular phosphate metabolism (Saiardi 2012a, b). The inositol phosphates are involved in some way in virtually every physiological process. In fact, several biological processes are specifically regulated by PP-IPs (for review see (Wundenberg and Mayr 2012; Wilson et al. 2013; Shears 2015; Thota and Bhandari 2015)). These include the ability to regulate cellular energetic status by controlling ATP levels (Szijgyartovc et al. 2011). Similarly, many specific biological functions have been attributed to polyP, and several chapters of this book are dedicated to these roles.

The objective of the current chapter is to highlight the metabolic and functional connection between PP-IPs and polyP, since in the yeast *Saccharomyces cerevisiae* these two classes of molecule share a strong metabolic relationship (Auesukaree et al. 2005; Lonetti et al. 2011). We mainly, but not exclusively, discuss the roles and interconnection of these two molecules in lower eukaryote the yeast and the amoeba *Dictyostelium discoideum*. Since the readership of book is primary polyP oriented, we should start by presenting some inositol phosphate background.

5.2 Introduction to Inositol Polyphosphates

The metabolism and physiological roles played by PP-IPs cannot be properly appreciated without introducing the inositol phosphates family of molecules. An extensive exploration of the inositol phosphates is not the objective of the current essay; thus we invite the interested reader to read the following review (Irvine and Schell

2001; Resnick and Saiardi 2008). Here we aim to give some historical background and the key facts about inositol phosphates.

In living organisms the inositol sugar is almost exclusively in its myo-inositol isomeric arrangement, possessing one single axial hydroxyl group on positions two and five remaining equatorial hydroxyl groups, in the same plane as the sugar ring (Fig. 5.1b). The combinatorial substitution of phosphate moieties for the six hydroxyls imparts complexity and functional significance to the inositol phosphates. Over 40 different inositol polyphosphates have been observed in diverse eukaryotic organisms resulting in a fairly busy metabolic map (Irvine and Schell 2001; Resnick and Saiardi 2008). The best characterised inositol phosphate is the calcium-release factor I(1,4,5)P_3 (IP_3) (Fig. 5.1b). The paradigm of receptor activation with subsequent phospholipase C (PLC)-mediated release of IP_3, followed by its binding to IP_3 receptor and the release of calcium from intracellular stores, represents one of the most classical signal transduction pathways. In this cascade IP_3 acts as the stereotypical 'second messenger' (Streb et al. 1983; Berridge et al. 2000; Irvine 2003).

After the establishment of IP_3 signalling paradigm by the middle 1980s, an era of proliferation and discovery of novel inositol polyphosphates began. A simplified inositol phosphate metabolic pathway leading to the synthesis of PP-IPs is depicted in Fig. 5.2. Over the following years, it became clear that not all inositol polyphosphates are signalling molecules. In many cases the newly identified inositol phosphates simply represent transient metabolites towards the synthesis of more complex inositol phosphates (Saiardi and Cockcroft 2008). Nevertheless, some inositol phosphates do exhibit specific signalling roles, including Ins(3,4,5,6)P_4, which regulates calcium-activated chloride (Cl^-) channel (Shears 2009b). The inositol

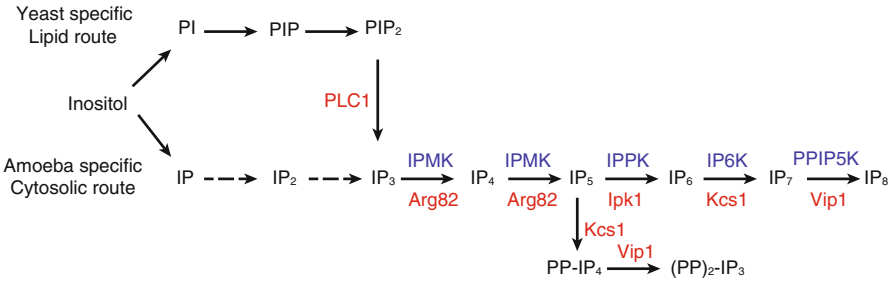

Fig. 5.2 Inositol pyrophosphate biosynthetic pathway. This simplified metabolic pathway illustrates the possible routes of inositol pyrophosphate biosynthesis starting from inositol. The yeast *S. cerevisiae* exclusively uses the lipid route to synthesise the PP-IPs derived from the fully phosphorylated ring of IP_6 or IP_7, or the inositol pyrophosphates derived from IP_5 or PP-IP_4. The lipid route requires the inositol to be converted into the lipid phosphoinositide phosphoinositol (PI), phosphoinositol phosphate (PIP) and phosphoinositol bisphosphate (PIP_2). The social amoeba *D. discoideum* utilises a direct cytosolic route to synthesise higher phosphorylated inositol species, the enzymology of which is not fully elucidated (*dashed line*). The names of the kinases catalysing each enzymatic step are indicated in *red* (yeast) and *blue* (mammalian). Phospholipase (PLC1), inositol polyphosphate multikinase (IPMK, Arg82), inositol pentakisphosphate 2-kinase (PPK1. Ipk1), inositol hexakisphosphate kinase (IP6K, Kcs1), diphosphoinositol pentakisphosphate kinase (PPIP5K, Vip1)

polyphosphate multikinase (IPMK in yeast known as Arg82, Ipk2) is the enzyme responsible for the synthesis of higher phosphorylated inositol phosphate, converting IP_3 to the inositol pentakisphosphate isomer I(1,3,4,5,6)P_5 (Saiardi et al. 1999, 2000b, 2001a; Odom et al. 2000). The formation of IP_5 represents the penultimate step in the synthesis of the most abundant inositol phosphate on earth, Ins(1,2,3,4,5,6)P_6 (thereafter called IP_6) also known as phytic acid or inositol hexakisphosphate. Its synthesis is achieved via the phosphorylation of IP_5's lone remaining axial hydroxyl by an IP_5 2-kinase (IPPK1 in yeast known as Ipk1) (York et al. 1999). The abundance of IP_6 in nature is owed largely to its use by plants for phosphate storage in seeds. In fact this molecule was originally discovered in seeds (Posternak 1919), hence the name phytic acid (Raboy 2003).

Even in non-plant eukaryotic cells, IP_6 is present at an intracellular concentration ranging from 10 to 100 μM, making it the most abundant inositol phosphate species in these cells too, often exceeding most other inositol phosphate by an order of magnitude or more (Shears 2001). Higher concentrations have been observed in the slime mould *D. discoideum* where the IP_6 concentrations can reach as high as 700 μM (Letcher et al. 2008; Pisani et al. 2014). Owing to its high charge density, IP_6, as polyP, is a strong chelator that readily forms insoluble salts with polyvalent cations. The formation of these complexes can result in the precipitation of IP_6 at higher concentrations. Under the cellular ionic conditions found in animal cells, soluble IP_6 concentrations are limited to <50 μM (likely in the form of a neutral, stable pentamagnesium salt) (Torres et al. 2005; Veiga et al. 2006). Thus, it is likely that much of cellular IP_6 is actually found in a 'bound state' or sequestered in vesicular compartments especially in slime mould.

In contrast to the rapid changes upon receptor activation of IP_3, IP_6 concentrations appear rather static, leading to the improper characterisation of IP_6 as lethargic molecule. However, the identification of IP_6 as a precursor to more phosphorylated inositol, the PP-IPs has revealed the true dynamic nature of cellular pool of IP_6. Although it has been more than 30 years since the definition of IP_3 as a second messenger, the PP-IPs continue to offer a different perspective to an already complex network of molecules (Burton et al. 2009; Shears 2009a).

5.3 Inositol Pyrophosphate Metabolism and Functions

The inositol pyrophosphates were identified in 1993 in *D. discoideum* and fluoride-treated pancreatoma cells (Menniti et al. 1993; Stephens et al. 1993). Subsequent studies confirmed the widespread evolutionary conservation of the PP-IPs. The best characterised of these 'high-energy' molecules are IP_7 (or PP-IP_5) and bis-diphosphoinositol tetrakisphosphate (IP_8, PP_2-IP_4) species (Fig. 5.1b). In most cell types, these species are present in submicromolar concentrations, representing 1–5 % of the total IP_6 levels. However, despite their low levels, the turnover of these molecules is remarkably rapid with up to 50 % of IP_6 cycling through the PP-IPs every hour in mammalian cells (Glennon and Shears 1993; Menniti et al. 1993). Thus, the cell invests a considerable amount of energy to maintain the PP-IPs steady-state

level; this is suggestive of a molecular switching property of the PP-IPs. Triphosphate-containing 'IP_8' species (PPP-IP_5) have also been observed (Draskovic et al. 2008). In addition to the inositol pyrophosphates derived from IP_6, inositol pyrophosphates can also be derived from the pyrophosphorylation of IP_5 and then called PP-IP_4 and $(PP)_2$-IP_3 (Fig. 5.2). These IP_5-derived PP-IPs are common constituents of mammalian cells where the cellular levels of IP_5 and IP_6 are similar (Wilson et al. 2013) and in the yeast *ipk1*Δ mutant that cannot synthesise IP_6 and thus accumulate IP_5 (Fig. 5.2).

The synthesis of PP-IPs has so far been attributed to two classes of enzymes, both conserved across the evolutionary spectrum. The IP_6-kinases (IP6Ks) synthesise IP_7 from IP_6 pyrophosphorylating the inositol ring on position five (Saiardi et al. 1999). These enzymes can also utilise IP_5 as substrate to generate PP-IP_4. The yeast genome encodes a single IP6K, called Kcs1 (Saiardi et al. 2000a), while mammals possess three isoforms called IP6K1 to 3 (Saiardi et al. 2000b). The second class of enzymes able to synthesise PP-IPs is the PP-IP5Ks (Choi et al. 2007; Mulugu et al. 2007). In vivo, these enzymes mainly convert IP_7 to IP_8 by pyrophosphorylating the inositol ring on position one. In vitro, this enzyme can also metabolise IP_6 although not IP_5. Yeast possesses a single PP-IP5K called Vip1 (Mulugu et al. 2007), while mammals possess two isoforms. The amoeba *D. discoideum* possesses a well-defined IP6K (gene called I6KA) (Luo et al. 2003) but as well other inositol pyrophosphate synthesising enzymes not yet fully characterised (Tom Livermore, AS unpublished result 2015). Furthermore, the amoeba also possesses a single PP-IP5K type of enzyme (Tom Livermore, AS unpublished result 2015).

PP-IPs can be dephosphorylated by at least two phosphatase activities. Oddly, one of these activities is attributed to PP-IP5K, which besides its kinase domain possesses an acid phosphatase domain (Pohlmann et al. 2014; Wang et al. 2015). This domain is able to dephosphorylate PP-IPs in position one, removing the pyrophosphate moiety added by the kinase domain of the same protein. This remarkable ability to generate and degrade pyrophosphate moiety in position one is an area of intense research. The second phosphatases able to degrade PP-IPs belong to the Nudix family of enzymes. Once more, yeast has a single member of this family called Ddp1. At least three have been identified in mammalian genomes, denoted DIPP1 to 3 (Safrany et al. 1998; Kilari et al. 2013). These enzymes have a broad substrate specificity and are also able to degrade nucleotide dimers such A6pA and polyP (see below) (Lonetti et al. 2011).

Both yeast and amoeba are powerful genetic experimental models; the deletion by homologous recombination of Kcs1 and I6KA gene, respectively, generates organism depleted of PP-IPs (Saiardi et al. 2002; Luo et al. 2003). The analysis of these mutants revealed many processes regulated by PP-IPs. In *D. discoideum* PP-IPs regulate chemotaxis (Luo et al. 2003), while the better characterised *kcs1*Δ reveals a key role of PP-IPs in vesicular trafficking (Saiardi et al. 2002), telomere length regulation (Saiardi et al. 2005; York et al. 2005), epigenetic transcriptional control (Burton et al. 2013; Worley et al. 2013), ribosomal biogenesis (Horigome et al. 2009; Thota et al. 2015) and cell wall stability (Dubois et al. 2002). This large variety of functions is suggestive that PP-IPs are controlling a very basic cellular

task alteration of which is manifested by this variety of phenotypes. The discovery that PP-IPs regulate yeast energetic metabolism, by repressing mitochondrial functionality and enhancing the glycolytic flux, gives an explanation to the diversity of phenotype attribute to PP-IPs (Wundenberg and Mayr 2012; Wilson et al. 2013). In fact the absence of PP-IPs in *kcs1*Δ yeast results in substantial elevated level of the central molecules of intermediate metabolism ATP (Szijgyartovc et al. 2011) that is virtually involved in any biochemical process taking place in living organism. It has been hypnotised that the ability of PP-IPs to regulate energy metabolism is manifested through their metabolic connection with polyP (Saiardi 2012b) as discussed below.

Intense debate surrounds the PP-IPs' molecular mechanism of action. As is the case for other inositol polyphosphates, binding/allosteric mechanisms for inositol pyrophosphate functionality have been proposed (Luo et al. 2003; Chakraborty et al. 2010). However, the high energetic potential of the β-phosphates in inositol pyrophosphates suggests a unique mechanism for this class of inositol phosphates. Indeed, early characterisation of IP_6-kinase demonstrated its capacity for ATP formation in the reverse by transferring a β-phosphate from IP_7 to ADP (Voglmaier et al. 1996). More importantly IP_7 appears to physiologically phosphorylate a variety of eukaryote protein targets. This ability allows IP_7 to transfer its β-phosphate to a prephosphorylated serine generating pyrophosphorylated-serine residue (Saiardi et al. 2004; Bhandari et al. 2007; Azevedo et al. 2009). Studying protein pyrophosphorylation has led to the discovery of a new protein post-translational modification directly mediated by polyP protein polyphosphorylation (Azevedo et al. 2015) described below.

5.4 Inorganic Polyphosphate Eukaryote Metabolism

Bacteria were the organisms where polyP metabolism was originally explored with the identification of the polyP biosynthetic enzyme in the protein polyphosphate kinase (PPK) (Ahn and Kornberg 1990; Zhang et al. 2002). Uniquely in eukaryotes *D. discoideum* possesses a polyP biosynthetic route of bacterial origin (Zhang et al. 2005, 2007). The amoeba genome contains a PPK enzyme homologous to the bacterial gene, called DdPPK1, acquired by horizontal gene transfer facilitated by the amoeba's bacterial feeding habit. This eukaryote PPK gene is present in a few related amoeba genomes belonging to the order of the Dictyosteliida; however it is unidentifiable by homology search in any other eukaryotic genome sequenced. For this reason there was great excitement when Kornberg's laboratory biochemically purified and identified a second amoeba polyphosphate kinase activity called DdPPK2 in an actin and actin-related protein complex (Gomez-Garcia and Kornberg 2004; Hooley et al. 2008). While homologues of these proteins are present in any eukaryote genome, the identification of the exact peptides responsible for DdPPK2 activity by characterisation of the recombinant protein has not been accomplished, casting serious doubt on the real nature of the original purified DdPPK2 activity. In fact, recent characterisation of *D. discoideum* DdPPK1 null mutant revealed that the

enzyme of bacterial origin is the only protein responsible for polyP synthesis in this amoeba (Livermore et al. 2016).

The second class of eukaryote where polyP synthesis has been elucidated is the yeast *S. cerevisiae*. In serendipitous fashion, the presence of polyP in the crystal structure of the cytosolic domain of subunit 4 of the vacuolar transporter chaperone complex (VTC) Vtc4 (or Phm3), which is important in regulating vacuolar membrane fusion (Muller et al. 2002), led to the discovery of an intrinsic polyphosphate polymerase activity (Hothorn et al. 2009). This vacuolar transporter chaperone complex consists of four subunits integrated into the vacuolar membrane; interestingly while the catalytic domain is located on the cytoplasmic side, polyP accumulates in the vacuolar lumen. Thus it has been proposed that the synthesis of polyP is coupled to its transport through a channel consisting of the VTC complex itself (Gerasimaite et al. 2014). The polyP translocation is dependent by the proton gradient generated by vacuolar membrane ATPase (V-ATPase or Vma2) since the *vma2*Δ mutant accumulates a reduce amount of polyP (Gerasimaite et al. 2014). Vtc4 homologous proteins, although absent in metazoa, can be identified in other protozoa such as in the order of Trypanosomatida (Lander et al. 2013). However, trypanosomes must possess a second enzyme able to synthesise polyP since the knockout of the Vtc4 homologous gene led to just a 20% reduction in polyP levels (Lander et al. 2013). The genome of metazoa lacks Vtc4 or PPK1 homologous counterparts; thus the synthesis of polyP in higher eukaryotes is an area of active investigation.

Two different classes of phosphatase degrade polyP: the exopolyphosphatases removing the phosphate from the polyP terminus, shortening the polyP polymer by one unit (Fig. 5.1a), and the endopolyphosphatases cleaving internal phosphoanhydride linkages, generating two smaller polyP molecules (Fig. 5.1b). A yeast exopolyphosphatase Ppx1 is a very active protein that progressively hydrolyses linear polyP chains longer than three phosphates, releasing free phosphate indispensable for metabolic processes and therefore regulating cellular phosphate homoeostasis. Ppx1 does not metabolise pyrophosphate, ATP, the cyclic form of tripolyphosphate or PP-IPs. Ppx1 is a member of the so-called DHH phosphoesterase family characterised by the aspartic acid (D) followed by two histidine (H) signatures in domain three of the protein (Aravind and Koonin 1998). Phosphatases possessing this DHH signature represent a group of functionally related enzymes that include pyrophosphatases. Yeast Ppx1 is usually localised in the cytoplasm; however mitochondrial matrix localisation has been also observed (Andreeva et al. 2008). In humans the Ppx1 homologue is called h-prune or simply Prune. The characterisation of its biochemical activity revealed that Prune is a short-chain exopolyphosphatase. Interestingly, Prune has been associated with metastatic cancer progression since it is a binding partner of the metastasis suppressor nm23-H1; thus it has been proposed that Prune's ability to metabolise polyP plays some kind of role in cancer (Tammenkoski et al. 2008).

The yeast endopolyphosphatase Ppn1 (also known as Phm5) cleaves long chains of polyP to generate shorter chains (Sethuraman et al. 2001). Purified Ppn1 is a homotetramer of a 35-kDa subunit that derives from the proteolytic cleavage of a

78-kDa polypeptide (674 amino acids). Although originally characterised as endopolyphosphatase, Ppn1 possesses both endo- and exopolyphosphatase activities. In the presence of magnesium, Ppn1 generates shorter chain polyP, while Co^{2+} stimulates free-phosphate release (Andreeva et al. 2015). The maturation by proteolytic cleavage and N-glycosylation is essential to obtain catalytically active Ppn1 proteins (Shi and Kornberg 2005). Thus the production of bacterially expressed recombinant Ppn1 has not been achieved. The need for an active and easy to express endopolyphosphatase is very important since there are pools of polyP that are not degraded by the single action of an exopolyphosphatase as Ppx1 (Wurst et al. 1995). Some polyPs appear to be protected possibly by the attachment of a protecting group at the polyP terminus (Kornberg et al. 1999).

A new yeast endopolyphosphatase, capable of being recombinantly expressed in bacteria, has been recently discovered in the gene named Ddp1 (Lonetti et al. 2011). This discovery is not surprising because endopolyphosphatase activity has been detected in the cytosol of the double mutant of *ppn1*Δ*ppx1*Δ (Lichko et al. 2008). Ddp1 stands for diadenosine and diphosphoinositol polyphosphate phosphohydrolase since this enzyme was originally characterised to degrade nucleotide analogues, such as diadenosine hexaphosphate (A6pA) and PP-IPs (Safrany et al. 1999). Thus Ddp1 is a multi-substrate phosphatase able to attack the phosphoanhydride bond of A6pA, PP-IPs and polyP (Lonetti et al. 2011). Ddp1 belongs to the Nudix (nucleoside diphosphate-linked moiety X) hydrolase family possessing a 23-amino acid catalytic domain denominated the MutT motif (McLennan 2006). In mammals, four putative Ddp1/DIPPs have been characterised: DIPP1, DIPP2, and DIPP3a/b. All four mammalian proteins present a robust polyP endopolyphosphohydrolase activity (Lonetti et al. 2011).

5.5 Inositol Pyrophosphate and Inorganic Polyphosphate Metabolic Connected

The fact that a common phosphatase Ddp1 (DIPPs in mammals) can metabolise both PP-IPs and polyP is indicative of a metabolic connection between these two classes of molecules. More compelling evidence of a metabolic link between polyP and PP-IPs came from genetic screening. Microarray analysis performed on *arg82*Δ and *kcs1*Δ strains revealed the constitutive expression of the secreted acid phosphatase Pho5 (El Alami et al. 2003). Accordingly, the *kcs1*Δ yeast strain was also shown to exhibit a reduced uptake of phosphate from the culture medium (Saiardi et al. 2004). Subsequently, a functional genome-wide screen, designed to identify secreted acid phosphatase activity, identified Plc1, Arg82 and Kcs1 as genes responsible for the constitutive expression of Pho5 (Auesukaree et al. 2005). By using ^{31}P-NMR to detect polyP, this work also identified the pathway of PP-IPs synthesis as essential for the accumulation of polyP (Auesukaree et al. 2005).

In apparent conflict, the yeast *ipk1*Δ strain possesses normal levels of polyP, and although it lacks IP_7, it does possess PP-IPs derived from IP_5 (Fig. 5.2); thus the authors concluded that these molecules, PP-IP_4 and/or $(PP)_2$-IP_3, regulate polyP

metabolism, not IP_7. However, systematic biochemical analysis of polyP by gel electrophoresis in a panel of PP-IP pathway mutants has revealed that any species of PP-IP is able to control the level of polyP (Lonetti et al. 2011). Furthermore, the dynamic turnover of PP-IPs is linked to polyP biosynthesis, with their levels fluctuating in tandem in response to manipulation of phosphate. The phosphate overplus assay, in which phosphorus is withdrawn and then resupplied to the growing medium of yeast, induces the reduction and subsequent resynthesis of both polyP and PP-IPs (Lonetti et al. 2011). Understanding how PP-IPs regulate polyP metabolism is an area of intense research (Saiardi 2012b), and since polyP is buffering cellular phosphates, this connection might have far-reaching implication.

In *D. discoideum*, preliminary analysis revealed that, to the contrary of yeast, the single deletion of IP6K does not result in a dramatic alteration of polyP metabolism (Tom Livermore, AS unpublished result 2015). However, while wild-type (WT) *D. discoideum* undergoes a dramatic developmental accumulation of polyP, the analysis of ppk1 null amoeba revealed a failure to accumulate polyP (Livermore et al. 2016). This strain revealed a compensatory increase in PP-IPs, demonstrating a reciprocal metabolic connection between PP-IPs and polyP (Livermore et al. 2016).

Nevertheless, the ability of PP-IPs to regulate polyP metabolism appears to be conserved in mammals since the analysis of the knockout *ip6k1*−/− mouse revealed a decrease in polyP abundance in blood platelet (Ghosh et al. 2013). Interestingly, regulation of mammalian phosphate homoeostasis, probably through polyP metabolism, has been associated to all the three mammalian IP6Ks. In fact, the IP6K2 was initially cloned while searching for a novel plasma membrane phosphate transporter and initially called Phosphate inorganic Uptake Stimulator (PiUS) (Norbis et al. 1997; Schell et al. 1999). Transfection of PiUS into Xenopus oocytes stimulated the uptake of radioactive phosphate, a result reminiscent of the reduced phosphate uptake of *kcs1*Δ yeast (Saiardi et al. 2004). Furthermore, single-nucleotide polymorphisms in the human IP6K3 promoter were identified as important for regulation of serum phosphate levels in humans (Kestenbaum et al. 2010). While the exact nature of the metabolic connection between PP-IPs and polyP/phosphate is still unclear, it appears obvious that deciphering this important link will help to clarify many PP-IPs and polyP attributes.

5.6 Inorganic Polyphosphate Function in *Saccharomyces cerevisiae*

The baker's yeast *S. cerevisiae* is a free-living organism, exposing it to an ever-changing phosphate environment. Thus, yeast has developed a sophisticated phosphate regulation pathway, known as the PHO regulon. This pathway controls the phosphate needs of the cell from membrane transport to transcriptional response (Ogawa et al. 2000; Korber and Barbaric 2014). In phosphate-rich media (environment), yeast accumulates as much phosphate as possible for use in the event of entering a low-phosphate environment. However, the cellular concentration of free

phosphate must remain fixed. Consequently, in phosphate-rich condition, phosphate is converted to the osmotically inert form of polyP.

Yeast primarily stores polyP inside the vacuolar lumen. This organelle possesses analogous characteristics of an acidocalcisome, being acidic and rich in calcium (Docampo et al. 2005; Gerasimaite et al. 2014). Besides in the vacuole polyP has been detected in the yeast cytosol, mitochondria and nucleus (Lichko et al. 2006a, b). The vacuolar accumulation of polyP is counterbalanced by the simultaneous accumulation of basic amino acid such as arginine and lysine as demonstrated in *Neurospora crassa* (Cramer and Davis 1984); however, the negative polyP charge has also been shown to be neutralised by bivalent cations, mainly calcium and spermidine (Kornberg et al. 1999). The compartmentalisation of polyP in the vacuole and the appropriate cytosolic neutralisation of its charge are essential to yeast well-being. In fact, the overexpression of *Escherichia coli* PPK enzyme in the yeast cytosol results in a marked toxicity (Gerasimaite et al. 2014).

While the discovery of Vtc4 as the yeast polyP synthesising enzymes is relatively recent (Ogawa et al. 2000; Hothorn et al. 2009), the VTC complex has been extensively studied. This complex has been shown to regulate vacuolar physiology, protein transport, membrane fusion and even microautophagy suggesting that some of these functions might be mediated by polyP. The null mutant of the endopolyphosphatase, *ppn1*Δ, accumulates long-chain polyP (Ogawa et al. 2000; Lonetti et al. 2011) and is defective in growth in minimal media. Meanwhile the double mutant of *ppn1*Δ*ppx1*Δ rapidly loses viability in stationary phase (Sethuraman et al. 2001), underlining a basic and generic role of polyP to maintain cell fitness.

5.7 Inorganic Polyphosphate Function in *Dictyostelium discoideum*

The experimental model organism *D. discoideum* was originally selected to study the development of multicellularity. This amoeba grows and replicates as a unicellular organism in favourable conditions. However, in stress conditions, it undergoes a developmental transition into a multicellular form. Upon starvation thousands of unicellular amoebas aggregate to form multicellular complexes, culminating in the formation of 'fruiting body', which is comprised of spore cells and stalk cells. The amoeba's simplicity and its remarkable developmental behaviour have led the study of biological processes from transcription to signalling in this organism (Franca-Koh et al. 2006; Muramoto et al. 2012; Pisani et al. 2014).

In addition, the polyP research field is amongst the many to have taken advantage of this organism. In the early 1970s, the presence of polyP in *D. discoideum* was recorded by the pioneering work of Gezelius (1974). By the 1980s, the use of 31P-NMR allowed the direct detection of polyP in the amoeba spore extract (Klein et al. 1988). Twenty years later, the Kornberg laboratory biochemically characterised the two enzymes, DdPPK1 and DdPPK2, described above. The analysis of a ppk1 null strain generated in the Kornberg laboratory revealed that polyP regulates amoeba development, predation and the late stages of cytokinesis (Zhang et al. 2005, 2007).

However, only about 3 % of PPK1 gene was deleted in this strain, and it cannot be excluded that a partially active DdPPK1 protein was still present in these cells.

Recently, a new *D. discoideum ppk1* mutant line was generated, this time deleting about 69 % of the Ppk1 gene. This new mutant revealed a defect in vegetative growth, development and spore germination (Livermore et al. 2016). The defective spore germination observed in the new *ppk1* mutant can be explained by the previously mentioned dramatic increase (more than 100-fold) in the level of polyP during development of *D. discoideum*. This accumulation of polyP occurs predominantly in the spore and plays a fundamental role during amoeba spore germination, as demonstrated by the reduction in germination efficiency in *ppk1* cells.

In dormant cells, polyP can be primarily considered as a phosphate storage and/or cation-sequestering molecule. Therefore, it seems likely that catabolism of polyP in the amoeba spore plays a key role in releasing these essential molecules in germinating spores. In fact, polyP accumulation in the amoeba spore is reminiscent of role played by IP_6 in plant seeds (Raboy 2003), where IP_6 accumulates and acts as both a phosphate and cation store during seed germination.

However, in vegetative *D. discoideum* cells, polyP must play a more dynamic role, since the general fitness of ppk1 amoebae is substantially reduced. The analysis of ppk1 demonstrated the dependence of cellular energetic metabolism on polyP, since a 2.5-fold decrease in cellular ATP level was recorded in vegetative ppk1 amoeba. The unexpected increase in the level of polyP after treating cells with mitochondrial poisons (Livermore et al. 2016) revealed in amoeba a not-yet-understood connection between polyP and energetic metabolism confirming mammalian observation (Abramov et al. 2007). The observed compensatory increase of inositol pyrophosphates and ATP during ppk1 development supports a model in which there is a functional interplay between inositol pyrophosphates, ATP and polyP (Livermore et al. 2016).

5.8 Protein Polyphosphorylation

The novel protein post-translational modification (PTM) lysine polyphosphorylation offers a new perspective on polyP research, representing an ideal molecular mechanism by which polyP might regulate protein activities. Protein polyphosphorylation was discovered while studying protein pyrophosphorylation (Saiardi et al. 2004; Bhandari et al. 2007; Azevedo et al. 2009). This latter PTM consists of the transfer of the β-phosphate of IP_7 to a prephosphorylated serine, generating a pyrophosphoserine residue. Therefore protein pyrophosphorylation must be directly dependent on PP-IPs' cellular level. Accordingly, it was noticed that an in vitro target of IP_7, Nsr1 (Saiardi et al. 2004) migrated on SDS-PAGE differently depending on the amount of IP_7 present (Azevedo et al. 2009). In kcs1Δ extracts, which have no detectable amounts of IP_7, Nsr1 migrated as a sharp band of ~67 kDa, while in WT extracts, Nsr1 showed a huge mobility shift with a smeary appearance migrating around ~130 kDa (Azevedo et al. 2015). Since, as discussed above, in yeast there is a direct correlation between the cellular levels of PP-IPs and polyP, it

was thought that the dramatic mobility shift of Nsr1 might correlate with the presence of both PP-IPs and polyP in WT yeast, and conversely with their absence in *kcs1*Δ. To test the possibility that polyP was mediating Nsr1 mobility shift, *vtc4*Δ extracts were analysed; these extracts are lacking polyP but possess normal level of IP_7. The observation that Nsr1 mobility on SDS-PAGE in *vtc4*Δ was similar to that in *kcs1*Δ indicated that the mobility shift of Nsr1 was directly dependent on the level of polyP and not on IP_7 levels (Azevedo et al. 2015). Interestingly, polyphosphorylation was shown to be non-enzymatic, resulting from a nucleophilic attack on an internal polyP phosphate ester linkage. Non-enzymatic PTMs are very common, for example, cysteine nitrosylation, and are often dependent on certain features: metabolic status, specific structural determinants and/or the local environment. Indeed, polyphosphorylation occurs at lysine residues embedded in an acidic, serine-rich domain called the PASK (for polyacidic serine and lysine rich) cluster and depends on the polyP cellular level. Interestingly lysine is the amino acid subjected to the highest variety of PTMs due to its chemical properties (Azevedo and Saiardi 2014). Another way to control non-enzymatic PTMs is by regulating their removal. The exopolyphosphatase Ppx1 has been shown to depolyphosphorylate Nsr1. Thus, regulating Ppx1 expression or subcellular localization will regulate protein polyphosphorylation signalling.

Besides Nsr1, topoisomerase Top1 was also shown to be polyphosphorylated. Polyphosphorylation regulates the ability of Nsr1 and Top1 to interact; when both proteins are polyphosphorylated, in WT yeast, they fail to interact. On the contrary in *kcs1*Δ or *vtc4*Δ yeast when both proteins are non-polyphosphorylated, they interact. Mechanistically, topoisomerase binds and relaxes supercoiled DNA. Like polyP DNA is a highly negatively charged molecule. We can envisage a mechanism in which polyphosphorylated Top1 mimics Top1 binding to DNA. Thus it has been proposed that polyphosphorylation is acting as a molecular switch preventing Top1 association with DNA (Azevedo and Saiardi 2014). Upon requirement, Top1 would be depolyphosphorylated and thus become available to associate with nucleic acids. Since the nucleus and nucleolus in particular appear to possess a well-defined set of acidic proteins (Kuehl et al. 1986), protein polyphosphorylation might represent an additional layer of regulation of nuclear signalling. In this model polyphosphorylation mimics nucleic acid association, keeping the enzyme away from DNA/RNA, and once depolyphosphorylated, the proteins can then associate with DNA/RNA and become active.

5.9 Concluding Remarks

The polymer polyP has for many years been considered a molecular fossil with minor if any real functions. Similarly, until recently PP-IPs have been considered an interesting curiosity and nothing more. Research into these molecules is gaining momentum, and both polyP and PP-IPs have now been established as important fields of research. Elucidating polyPs' and PP-IPs' physiological roles may not only lead to the uncovering of fundamental processes, such as the molecular basis of phosphate homoeostasis, but could help combat human diseases. Both molecules

have in fact been associated with cancer and metabolic disorders. To reach these goals, research using the yeast and the amoeba will be of foremost importance; many major polyP and PP-IPs discoveries are likely still to come from working with these simple eukaryotes.

Acknowledgements The author would like to thank Drs. T. Livermore and M. Wilson for promptly reading the manuscript and the members of the laboratory for their constructive inputs.

Funding This work was supported by the Medical Research Council (MRC) core support to the MRC/UCL Laboratory for Molecular Cell Biology University Unit (MC_UU_1201814).

References

Abramov AY, Fraley C, Diao CT et al (2007) Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death. Proc Natl Acad Sci U S A 104(46):18091–18096

Ahn K, Kornberg A (1990) Polyphosphate kinase from *Escherichia coli*. Purification and demonstration of a phosphoenzyme intermediate. J Biol Chem 265(20):11734–11739

Andreeva NA, Kulakovskaya TV, Kulakovskaya EV et al (2008) Polyphosphates and exopolyphosphatases in cytosol and mitochondria of *Saccharomyces cerevisiae* during growth on glucose or ethanol under phosphate surplus. Biochemistry (Mosc) 73(1):65–69

Andreeva N, Trilisenko L, Eldarov M et al (2015) Polyphosphatase PPN1 of *Saccharomyces cerevisiae*: switching of exopolyphosphatase and endopolyphosphatase activities. PLoS One 10(3):e0119594

Aravind L, Koonin EV (1998) A novel family of predicted phosphoesterases includes Drosophila prune protein and bacterial RecJ exonuclease. Trends Biochem Sci 23(1):17–19

Auesukaree C, Tochio H, Shirakawa M et al (2005) Plc1p, Arg82p, and Kcs1p, enzymes involved in inositol pyrophosphate synthesis, are essential for phosphate regulation and polyphosphate accumulation in *Saccharomyces cerevisiae*. J Biol Chem 280(26):25127–25133

Azevedo C, Saiardi A (2014) Functions of inorganic polyphosphates in eukaryotic cells: a coat of many colours. Biochem Soc Trans 42(1):98–102

Azevedo C, Burton A, Ruiz-Mateos E et al (2009) Inositol pyrophosphate mediated pyrophosphorylation of AP3B1 regulates HIV-1 Gag release. Proc Natl Acad Sci U S A 106(50):21161–21166

Azevedo C, Livermore T, Saiardi A (2015) Protein polyphosphorylation of lysine residues by inorganic polyphosphate. Mol Cell 58(1):71–82

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1(1):11–21

Bhandari R, Saiardi A, Ahmadibeni Y et al (2007) Protein pyrophosphorylation by inositol pyrophosphates is a posttranslational event. Proc Natl Acad Sci U S A 104(39):15305–15310

Burton A, Hu X, Saiardi A (2009) Are inositol pyrophosphates signalling molecules? J Cell Physiol 220(1):8–15

Burton A, Azevedo C, Andreassi C et al (2013) Inositol pyrophosphates regulate JMJD2C-dependent histone demethylation. Proc Natl Acad Sci U S A 110(47):18970–18975

Chakraborty A, Koldobskiy MA, Bello NT et al (2010) Inositol pyrophosphates inhibit Akt signaling, thereby regulating insulin sensitivity and weight gain. Cell 143(6):897–910

Choi JH, Williams J, Cho J et al (2007) Purification, sequencing, and molecular identification of a mammalian PP-InsP5 kinase that is activated when cells are exposed to hyperosmotic stress. J Biol Chem 282(42):30763–30775

Cramer CL, Davis RH (1984) Polyphosphate-cation interaction in the amino acid-containing vacuole of *Neurospora crassa*. J Biol Chem 259(8):5152–5157

Docampo R, de Souza W, Miranda K et al (2005) Acidocalcisomes – conserved from bacteria to man. Nat Rev Microbiol 3(3):251–261

Draskovic P, Saiardi A, Bhandari R et al (2008) Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups. Chem Biol 15(3):274–286

Dubois E, Scherens B, Vierendeels F et al (2002) In *Saccharomyces cerevisiae*, the inositol polyphosphate kinase activity of Kcs1p is required for resistance to salt stress, cell wall integrity, and vacuolar morphogenesis. J Biol Chem 277(26):23755–23763

El Alami M, Messenguy F, Scherens B et al (2003) Arg82p is a bifunctional protein whose inositol polyphosphate kinase activity is essential for nitrogen and PHO gene expression but not for Mcm1p chaperoning in yeast. Mol Microbiol 49(2):457–468

Faxalv L, Boknas N, Strom JO et al (2013) Putting polyphosphates to the test: evidence against platelet-induced activation of factor XII. Blood 122(23):3818–3824

Franca-Koh J, Kamimura Y, Devreotes P (2006) Navigating signaling networks: chemotaxis in *Dictyostelium discoideum*. Curr Opin Genet Dev 16(4):333–338

Gerasimaite R, Sharma S, Desfougeres Y et al (2014) Coupled synthesis and translocation restrains polyphosphate to acidocalcisome-like vacuoles and prevents its toxicity. J Cell Sci 127(Pt 23):5093–5104

Gezelius K (1974) Inorganic polyphosphates and enzymes of polyphosphate metabolism in the cellular slime mold *Dictyostelium discoideum*. Arch Microbiol 98(4):311–329

Ghosh S, Shukla D, Suman K et al (2013) Inositol hexakisphosphate kinase 1 maintains hemostasis in mice by regulating platelet polyphosphate levels. Blood 122(8):1478–1486

Glennon MC, Shears SB (1993) Turnover of inositol pentakisphosphates, inositol hexakisphosphate and diphosphoinositol polyphosphates in primary cultured hepatocytes. Biochem J 293(Pt 2):583–590

Gomez-Garcia MR, Kornberg A (2004) Formation of an actin-like filament concurrent with the enzymatic synthesis of inorganic polyphosphate. Proc Natl Acad Sci U S A 101(45):15876–15880

Gray MJ, Wholey WY, Wagner NO et al (2014) Polyphosphate is a primordial chaperone. Mol Cell 53(5):689–699

Hooley P, Whitehead MP, Brown MR (2008) Eukaryote polyphosphate kinases: is the 'Kornberg' complex ubiquitous? Trends Biochem Sci 33(12):577–58

Horigome C, Ikeda R, Okada T et al (2009) Genetic interaction between ribosome biogenesis and inositol polyphosphate metabolism in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 73(2):443–446

Hothorn M, Neumann H, Lenherr ED et al (2009) Catalytic core of a membrane-associated eukaryotic polyphosphate polymerase. Science 324(5926):513–516

Irvine RF (2003) 20 years of Ins(1,4,5)P3, and 40 years before. Nat Rev Mol Cell Biol 4(7):586–590

Irvine RF, Schell MJ (2001) Back in the water: the return of the inositol phosphates. Nat Rev Mol Cell Biol 2(5):327–338

Jahid IK, Silva AJ, Benitez JA (2006) Polyphosphate stores enhance the ability of *Vibrio cholerae* to overcome environmental stresses in a low-phosphate environment. Appl Environ Microbiol 72(11):7043–7049

Kestenbaum B, Glazer NL, Kottgen A et al (2010) Common genetic variants associate with serum phosphorus concentration. J Am Soc Nephrol 21(7):1223–1232

Kilari RS, Weaver JD, Shears SB et al (2013) Understanding inositol pyrophosphate metabolism and function: kinetic characterization of the DIPPs. FEBS Lett 587(21):3464–3470

Klein G, Cotter DA, Martin JB et al (1988) Germination of *Dictyostelium discoideum* spores. A phosphorus-31 NMR analysis. Biochemistry 27(21):8199–8203

Korber P, Barbaric S (2014) The yeast PHO5 promoter: from single locus to systems biology of a paradigm for gene regulation through chromatin. Nucleic Acids Res 42(17):10888–10902

Kornberg A, Rao NN, Ault-Riche D (1999) Inorganic polyphosphate: a molecule of many functions. Annu Rev Biochem 68:89–125
Kuehl L, Childers TJ, McCauley RM (1986) The occurrence of extended acidic sequences in non-histone chromosomal proteins. Arch Biochem Biophys 248(1):272–281
Lander N, Ulrich PN, Docampo R (2013) *Trypanosoma brucei* vacuolar transporter chaperone 4 (TbVtc4) is an acidocalcisome polyphosphate kinase required for *in vivo* infection. J Biol Chem 288(47):34205–34216
Letcher AJ, Schell MJ, Irvine RF (2008) Do mammals make all their own inositol hexakisphosphate? Biochem J 416(2):263–270
Lichko L, Kulakovskaya T, Pestov N et al (2006a) Inorganic polyphosphates and exopolyphosphatases in cell compartments of the yeast *Saccharomyces cerevisiae* under inactivation of PPX1 and PPN1 genes. Biosci Rep 26(1):45–54
Lichko LP, Kulakovskaya TV, Kulaev IS (2006b) Inorganic polyphosphate and exopolyphosphatase in the nuclei of *Saccharomyces cerevisiae*: dependence on the growth phase and inactivation of the PPX1 and PPN1 genes. Yeast 23(10):735–740
Lichko LP, Kulakovskaya TV, Kulakovskaya EV et al (2008) Inactivation of PPX1 and PPN1 genes encoding exopolyphosphatases of *Saccharomyces cerevisiae* does not prevent utilization of polyphosphates as phosphate reserve. Biochemistry (Mosc) 73(9):985–989
Livermore TM, Chubb JR, Saiardi A (2016) Developmental accumulation of polyphosphate affects germination and energetic metabolism in *D. discoideum*. Proc Natl Acad Sci U S A. doi:10.1073/pnas.1519440113
Lonetti A, Szijgyarto Z, Bosch D et al (2011) Identification of an evolutionarily conserved family of inorganic polyphosphate endopolyphosphatases. J Biol Chem 286(37):31966–31974
Losito O, Szijgyarto Z, Resnick AC et al (2009) Inositol pyrophosphates and their unique metabolic complexity: analysis by gel electrophoresis. PLoS One 4(5):e5580
Luo HR, Huang YE, Chen JC et al (2003) Inositol pyrophosphates mediate chemotaxis in Dictyostelium via pleckstrin homology domain-PtdIns(3,4,5)P3 interactions. Cell 114(5):559–572
McLennan AG (2006) The Nudix hydrolase superfamily. Cell Mol Life Sci 63(2):123–143
Menniti FS, Miller RN, Putney JW et al (1993) Turnover of inositol polyphosphate pyrophosphates in pancreatoma cells. J Biol Chem 268(6):3850–3856
Moreno SN, Docampo R (2013) Polyphosphate and its diverse functions in host cells and pathogens. PLoS Pathog 9(5):e1003230
Morrissey JH, Choi SH, Smith SA (2012) Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation. Blood 119(25):5972–5979
Muller O, Bayer MJ, Peters C et al (2002) The Vtc proteins in vacuole fusion: coupling NSF activity to V(0) trans-complex formation. EMBO J 21(3):259–269
Muller F, Mutch NJ, Schenk WA et al (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators *in vivo*. Cell 139(6):1143–1156
Mulugu S, Bai W, Fridy PC et al (2007) A conserved family of enzymes that phosphorylate inositol hexakisphosphate. Science 316(5821):106–109
Muramoto T, Cannon D, Gierlinski M et al (2012) Live imaging of nascent RNA dynamics reveals distinct types of transcriptional pulse regulation. Proc Natl Acad Sci U S A 109(19):7350–7355
Norbis F, Boll M, Stange G et al (1997) Identification of a cDNA/protein leading to an increased P_i-uptake in *Xenopus laevis* oocytes. J Membr Biol 156(1):19–24
Odom AR, Stahlberg A, Wente SR et al (2000) A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. Science 287(5460):2026–2029
Ogawa N, DeRisi J, Brown PO (2000) New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol Biol Cell 11(12):4309–4321
Pisani F, Livermore T, Rose G et al (2014) Analysis of *Dictyostelium discoideum* inositol pyrophosphate metabolism by gel electrophoresis. PLoS One 9(1), e85533

Pohlmann J, Risse C, Seidel C et al (2014) The Vip1 inositol polyphosphate kinase family regulates polarized growth and modulates the microtubule cytoskeleton in fungi. PLoS Genet 10(9):e1004586

Posternak S (1919) Sur la synthése de l'ether hexaphosphorique de l'inosite avec le principe phosphoorganique de réserve des plantes vertes. C R Acad Sci 169:138–140

Raboy V (2003) myo-Inositol-1,2,3,4,5,6-hexakisphosphate. Phytochemistry 64(6):1033–1043

Rao NN, Gomez-Garcia MR, Kornberg A (2009) Inorganic polyphosphate: essential for growth and survival. Annu Rev Biochem 78:605–647

Resnick AC, Saiardi A (2008) Inositol polyphosphate multikinase: metabolic architect of nuclear inositides. Front Biosci 13:856–866

Safrany ST, Caffrey JJ, Yang X et al (1998) A novel context for the 'MutT' module, a guardian of cell integrity, in a diphosphoinositol polyphosphate phosphohydrolase. EMBO J 17(22):6599–6607

Safrany ST, Ingram SW, Cartwright JL et al (1999) The diadenosine hexaphosphate hydrolases from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* are homologues of the human diphosphoinositol polyphosphate phosphohydrolase. Overlapping substrate specificities in a MutT-type protein. J Biol Chem 274(31):21735–21740

Saiardi A (2012a) Cell signalling by inositol pyrophosphates. Subcell Biochem 59:413–443

Saiardi A (2012b) How inositol pyrophosphates control cellular phosphate homeostasis? Adv Biol Regul 52(2):351–359

Saiardi A, Cockcroft S (2008) Human ITPK1: a reversible inositol phosphate kinase/phosphatase that links receptor-dependent phospholipase C to Ca^{2+}-activated chloride channels. Sci Signal 1(4):pe5

Saiardi A, Erdjument-Bromage H, Snowman AM et al (1999) Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr Biol 9(22):1323–1326

Saiardi A, Caffrey JJ, Snyder SH et al (2000a) The inositol hexakisphosphate kinase family. Catalytic flexibility and function in yeast vacuole biogenesis. J Biol Chem 275(32):24686–24692

Saiardi A, Caffrey JJ, Snyder SH et al (2000b) Inositol polyphosphate multikinase (ArgRIII) determines nuclear mRNA export in *Saccharomyces cerevisiae*. FEBS Lett 468(1):28–32

Saiardi A, Nagata E, Luo HR et al (2001a) Mammalian inositol polyphosphate multikinase synthesizes inositol 1,4,5-trisphosphate and an inositol pyrophosphate. Proc Natl Acad Sci U S A 98(5):2306–2311

Saiardi A, Nagata E, Luo HR et al (2001b) Identification and characterization of a novel inositol hexakisphosphate kinase. J Biol Chem 276(42):39179–39185

Saiardi A, Sciambi C, McCaffery JM et al (2002) Inositol pyrophosphates regulate endocytic trafficking. Proc Natl Acad Sci U S A 99(22):14206–14211

Saiardi A, Bhandari R, Resnick AC et al (2004) Phosphorylation of proteins by inositol pyrophosphates. Science 306(5704):2101–2105

Saiardi A, Resnick AC, Snowman AM et al (2005) Inositol pyrophosphates regulate cell death and telomere length through phosphoinositide 3-kinase-related protein kinases. Proc Natl Acad Sci U S A 102(6):1911–1914

Schell MJ, Letcher AJ, Brearley CA et al (1999) PiUS (Pi uptake stimulator) is an inositol hexakisphosphate kinase. FEBS Lett 461(3):169–172

Seidlmayer LK, Blatter LA, Pavlov E et al (2012) Inorganic polyphosphate-an unusual suspect of the mitochondrial permeability transition mystery. Channels (Austin) 6(6):463–467

Sethuraman A, Rao NN, Kornberg A (2001) The endopolyphosphatase gene: essential in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 98(15):8542–8547

Shears SB (2001) Assessing the omnipotence of inositol hexakisphosphate. Cell Signal 13(3):151–158

Shears SB (2009a) Diphosphoinositol polyphosphates: metabolic messengers? Mol Pharmacol 76(2):236–252

Shears SB (2009b) Molecular basis for the integration of inositol phosphate signaling pathways via human ITPK1. Adv Enzym Regul 49(1):87–96
Shears SB (2015) Inositol pyrophosphates: why so many phosphates? Adv Biol Regul 57:203–216
Shi X, Kornberg A (2005) Endopolyphosphatase in *Saccharomyces cerevisiae* undergoes post-translational activations to produce short-chain polyphosphates. FEBS Lett 579(9):2014–2018
Stephens L, Radenberg T, Thiel U et al (1993) The detection, purification, structural characterization, and metabolism of diphosphoinositol pentakisphosphate(s) and bisdiphosphoinositol tetrakisphosphate(s). J Biol Chem 268(6):4009–4015
Streb H, Irvine RF, Berridge MJ et al (1983) Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306(5938):67–69
Szijgyartovc Z, Garedew A, Azevedo C et al (2011) Influence of inositol pyrophosphates on cellular energy dynamics. Science 334(6057):802–805
Tammenkoski M, Koivula K, Cusanelli E et al (2008) Human metastasis regulator protein H-prune is a short-chain exopolyphosphatase. Biochemistry 47(36):9707–9713
Thota SG, Bhandari R (2015) The emerging roles of inositol pyrophosphates in eukaryotic cell physiology. J Biosci 40(3):593–605
Thota SG, Unnikannan CP, Thampatty SR et al (2015) Inositol pyrophosphates regulate RNA polymerase I-mediated rRNA transcription in *Saccharomyces cerevisiae*. Biochem J 466(1):105–114
Torres J, Dominguez S, Cerda MF et al (2005) Solution behaviour of myo-inositol hexakisphosphate in the presence of multivalent cations. Prediction of a neutral pentamagnesium species under cytosolic/nuclear conditions. J Inorg Biochem 99(3):828–840
Veiga N, Torres J, Dominguez S et al (2006) The behaviour of myo-inositol hexakisphosphate in the presence of magnesium(II) and calcium(II): protein-free soluble $InsP_6$ is limited to 49 microM under cytosolic/nuclear conditions. J Inorg Biochem 100(11):1800–1810
Voglmaier SM, Bembenek ME, Kaplin AI et al (1996) Purified inositol hexakisphosphate kinase is an ATP synthase: diphosphoinositol pentakisphosphate as a high-energy phosphate donor. Proc Natl Acad Sci U S A 93(9):4305–4310
Wang H, Nair VS, Holland AA et al (2015) Asp1 from *Schizosaccharomyces pombe* binds a [2Fe-2S](2+) cluster which inhibits inositol pyrophosphate 1-phosphatase activity. Biochemistry 54(42):6462–6474
Wilson MS, Livermore TM, Saiardi A (2013) Inositol pyrophosphates: between signalling and metabolism. Biochem J 452(3):369–379
Worley J, Luo X, Capaldi AP (2013) Inositol pyrophosphates regulate cell growth and the environmental stress response by activating the HDAC Rpd3L. Cell Rep 3(5):1476–1482
Wundenberg T, Mayr GW (2012) Synthesis and biological actions of diphosphoinositol phosphates (inositol pyrophosphates), regulators of cell homeostasis. Biol Chem 393(9):979–998
Wurst H, Shiba T, Kornberg A (1995) The gene for a major exopolyphosphatase of *Saccharomyces cerevisiae*. J Bacteriol 177(4):898–906
York JD, Odom AR, Murphy R et al (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science 285(5424):96–100
York SJ, Armbruster BN, Greenwell P et al (2005) Inositol diphosphate signaling regulates telomere length. J Biol Chem 280(6):4264–4269
Zakharian E, Thyagarajan B, French RJ et al (2009) Inorganic polyphosphate modulates TRPM8 channels. PLoS One 4(4):e5404
Zhang H, Ishige K, Kornberg A (2002) A polyphosphate kinase (PPK2) widely conserved in bacteria. Proc Natl Acad Sci U S A 99(26):16678–16683
Zhang H, Gomez-Garcia MR, Brown MR et al (2005) Inorganic polyphosphate in *Dictyostelium discoideum*: influence on development, sporulation, and predation. Proc Natl Acad Sci U S A 102(8):2731–2735
Zhang H, Gomez-Garcia MR, Shi X et al (2007) Polyphosphate kinase 1, a conserved bacterial enzyme, in a eukaryote, *Dictyostelium discoideum*, with a role in cytokinesis. Proc Natl Acad Sci U S A 104(42):16486–16491

Part II

Higher Eukaryotes

Methods of Inorganic Polyphosphate (PolyP) Assay in Higher Eukaryotic Cells

6

Maria E. Solesio and Evgeny V. Pavlov

Abstract

Experimental investigation of polyP in higher eukaryotic organisms is a very challenging task. Levels of polyP in higher eukaryotes have several orders of magnitude lower compared to the levels of polyP in bacteria. Although many methods that were developed for studies of polyP in bacteria can be adapted for eukaryotes, some important points need to be considered in order to allow investigation of polyP in low abundance situations. In this chapter, we present an overview of the experimental methods of polyP assays in higher eukaryotes that are currently available. These methods include the biochemical approaches of polyP extraction and quantification as well as fluorescent imaging approaches that are suitable for polyP assays in the live tissue culture cells. We discuss advantages and limitations of each of the approaches. Finally, we describe the use of bacterial polyP metabolising enzymes as a tool for studies of polyP in eukaryotic cells.

6.1 Introduction

Inorganic polyphosphate (polyP) assay in biological samples has always been a very challenging experimental problem. The very nature of the polymer makes it difficult to develop a method that will be selective and that will allow to accurately estimate the level of polyP in living organisms. There are two key challenges to the problem:

M.E. Solesio • E.V. Pavlov (✉)
Department of Basic Sciences, New York University College of Dentistry,
345 East 24th Street, Schwartz Building, Room 1030, New York, NY 10010, USA
e-mail: ep37@nyu.edu

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_6

First, polyP is a polymer made of many orthophosphates linked together by phosphoanhydride bonds in a way similar to ones found in ATP. The reported chain length of polyP polymer is in the range of several phosphate units to several hundreds of phosphate units (Kulaev et al. 2004). Taking into account that polyP conformation is highly variable and that this polymer does not have any specific chemical group in its structure, it is very difficult to develop a probe that can recognise polyP, but at the same time, does not react with any other molecules in the living cell.

Second, polyP is highly negatively charged polymer. This makes polyP a highly reactive polymer which can readily interact with divalent ions. Further, polyP has been reported to be able to form stable complexes with other biological polymers like RNA, polyhydroxybutyrate (PHB) and proteins (Kornberg et al. 1999). For this reason, even if a probe that can specifically bind to polyP is used in the assay, the presence of only free polyP in the biological sample or in living cell can be estimated. The pool of polyP that is complexed with other molecules will not be available to the probe. To some extent, the parallel can be made to the use of calcium-sensitive probe Fura-2 in calcium signalling assays when only free calcium, which makes up less than 1 % of the total calcium in the cell, is measured. This might be very practical for studies of the signalling properties, but not useful for measurements of the total levels of the molecule.

Compared to the unicellular organisms, in case of higher eukaryotic cells, the task of polyP detection and assay is complicated by the fact that in these cells, the levels of polyP are order of magnitudes lower compared to bacteria and yeast (Kumble and Kornberg 1995). Further, as many biological processes in eukaryotes are compartmentalised, it is highly desirable to perform polyP assays at the specific location, rather than measure overall levels of polyP in the whole cell. So far, specific pools of polyP were found in a number of subcellular locations including nuclei, acidocalcisomes, mitochondria, cell membranes and lysosomes (Kumble and Kornberg 1995; Moreno-Sanchez et al. 2012; Pavlov et al. 2010; Reusch 1989; Ruiz et al. 2004). Despite the great deal of difficulties associated with polyP assay methods, several useful approaches have been developed, and some of them are applied to the studies of higher eukaryotic cells and organisms. Here, we will review the use of these methods with the focus of assays of polyP in the higher eukaryotes. We will discuss key advantages and limitations of each of the methods as well as give specific examples on what kind of specific information regarding the polyP can be obtained by their use.

6.2 Overview of the Experimental Approaches for Investigation of PolyP

Many of the methods used to detect and to quantify biological polyP have been described in a number of reviews. These methods involve the use of fluorometric assays using DAPI and toluidine blue, NMR as well as electron microscopy

approaches (reviewed in Kulaev et al. (2004)). In the case of eukaryotes, two experimental strategies can be used. The first strategy is to extract and purify polyP from the tissue, following by the quantification of the purified material. The second strategy involves an attempt to measure polyP in situ, preferably in living cells. Ideally, both of these complimentary strategies should be used for accurate assay of polyP. However, depending on the specific research question, one or the other method is preferable.

6.3 Extraction and Quantification of PolyP in Eukaryotic Cells and Tissues

In many cases, it is desirable to quantify polyP located in the eukaryotic cell. The most challenging step during this process is the need to reliably extract polyP from these cells. This process is considerably more challenging compared to the polyP extraction from the bacterial and yeast cells, due to the much lower levels of polyP. Low levels of polyP can potentially lead to the loss of this polymer during the extraction process. Several methods have been reported to allow extraction of polyP. Most typical examples of purification protocol include polyP extraction and precipitation using acids and organic solvents (Clark et al. 1986). Another practical protocol includes the use of purification columns (Werner et al. 2007). Systematic comparison of the efficiency of the various extraction methods has not been performed which makes it very difficult to predict which methods are preferable in specific experiments. Likely, in each case, the comparison of several approaches needs to be done in order to conclude the best applicable method for a particular tissue. For example, in our experiments with mammalian cells with low abundance of polyP, we found the use of the glassmilk method, which was originally developed in Kornberg's lab for use in bacteria (Ault-Riche et al. 1998), produces the best results. In this approach, polyP is incubated with the glass beads. Due to its high affinity to the glass, polyP becomes absorbed by the beads and later can be extracted into the solution. The use of this method has allowed us to reliably detect polyP in mammalian mitochondria of various tissues (Pavlov et al. 2010; Seidlmayer et al. 2012) as well as in synaptosomes from brain (Stotz et al. 2014).

Once polyP is extracted, it needs to be quantified. The process of the assay of polyP following the extraction has been significantly improved after the discovery of the specific polyP metabolising enzymes. The two most commonly used enzymes are polyP kinase (PPK) and polyP phosphatase (PPX). PPK enzyme can stimulate the polyP production using ATP as a substrate. Since this reaction is reversible, the addition of excess amounts of ADP leads to the production of ATP, using polyP as a substrate. If polyP is present in the reaction mixture, then its amount can be quantified by the measurements of produced ATP. This method is highly selective, and in fact, the use of this method allowed to prove conclusively that polyP is indeed present in the mammalian cells (Kumble and Kornberg 1995). An alternative method relies on the use of PPX enzyme. This enzyme can hydrolyse polyP and produce single orthophosphate residues which can be quantified. While PPX method is also

highly selective, it has some limitations in its use. PPX enzyme cannot hydrolyse polyP of a short chain length. Notably, if only long-chain polyP is present in the initial sample, PPX is capable to hydrolyse it completely, suggesting that certain length of polyP is required for the initial enzyme binding to the polyP rather to its hydrolytic activity. Another limitation is that PPX cannot hydrolyse polyP with protected end groups (Kornberg et al. 1999). Interestingly, when compared directly, PPX and PPK assays showed variable specificity depending on the polyP chain length with PPX that is able to measure polyP as short as three units, while for PPK the cut-off was at the level of 40 units (Ohtomo et al. 2008). It has also been demonstrated that the activity of PPX is inhibited by the presence of RNA. Since RNA is commonly found to be present in polyP extracts, this has to be taken into consideration. Perhaps, one of the most important approaches that have to be considered to partially overcome these limitations is the use of an endopolyphosphatase (PPN) enzyme (Sethuraman et al. 2001) in combination with PPX. Unlike PPX, which cleaves orthophosphates from one end of the polyP, PPN enzyme "cuts" polyP in two pieces. Thus, if end groups of polyP are protected, PPN will produce a shorter version of polyP with at least one of the end groups of the polymer available to react with the PPX. Combination of PPN and PPX in the reaction mixture can allow to complete hydrolysis of polyP in the sample which amount can be quantified by the amount of orthophosphate released from polyP during the enzymatic reaction.

Another enzyme that can be used in place of PPX is alkaline phosphatase, which is a potent exopolyphosphatase (Lorenz and Schroder 2001). The advantage of the use of alkaline phosphatase is its high enzymatic activity. This enzyme is easily available from commercial sources. The limitation of the use of the alkaline phosphatase is its relatively low selectivity. Thus, it is preferable if it is used for polyP quantification in the preparations which do not have other known substrates for this enzyme.

Finally, the most simple but the least selective method relies on acid hydrolysis of polyP sample. This method relies on the fact that phosphoanhydride bound in polyP polymer can be hydrolysed under acidic conditions (Seidlmayer et al. 2012). Thus, the incubation of the sample in the acidic media followed by an orthophosphate assay allows the quantification of the polymer. This method could potentially produce a non-specific signal. However, it allows the complete hydrolysis of polyP in the sample, which can be an advantage compared to more selective methods that can leave some polyP intact.

6.4 Sizing of the Extracted PolyP

In addition to the quantification of the extracted polyP, another critical parameter of the polymer that needs to be measured is the size of this polymer. Sizing of polyP can best be achieved by performing the gel electrophoresis and comparing the migration rate with the rates of polyP standards of known length (Clark and Wood 1987). It should be noted that this approach requires the use of gel staining, which can be relatively non-specific. Thus, it has to be preferably used in combination

with the enzymatic approach that was discussed above. This will ensure that the origin of the bands seen on the gel is indeed related to the presence of polyP, but not to some other polyanions. In many cases, the gel staining is done by the use of toluidine blue dye (Clark and Wood 1987). Recently, a novel method which allows the use of DAPI for polyP gel sizing has been developed. This method is slightly more technically demanding, but it shows higher selectivity, compared to the toluidine blue method (Smith and Morrissey 2007). Most importantly, DAPI staining protocols demonstrate several orders of magnitude higher sensitivity (in terms of polyP detection limit), and thus they are particularly useful in experiments with low amounts of polyP, which is most often the case for mammalian samples.

6.5 Extraction and Assessment of the Complexed PolyP

Generally, protocols that were discussed above are applied for the assessment of polyP, which is present in the living cells and can be readily extracted. Similarly, it is possible to extract polyP, which is bound to such molecules as RNA or divalent cations. However, it should be taken into consideration that some pools of polyP might not be extracted by these methods and will require case-specific approaches. One example of such an approach is the detection of the pool of polyP complexed with PHB and calcium (channel forming polyP/Ca^{2+}/PHB complex). In this case, prior to polyP extraction, intact polyP/Ca^{2+}/PHB complex needs to be purified from the tissue by several stages of methanol-acetone precipitation, which is followed by chloroform extraction (Pavlov et al. 2005; Reusch 1989). Another example is the detection of polyP associated with proteins, for example, Ca^{2+}-ATPase and TRPM8 channel proteins (Reusch et al. 1997; Zakharian et al. 2009). In case of TRPM8 channel, which is a complex of proteins associated with PHB and polyP, purified TRPM8 protein can be applied in native gel electrophoresis and polyP associated with this protein can be visualised using direct toluidine blue staining (Zakharian et al. 2009). Notably, although toluidine blue is a relatively non-specific dye its use in combination with PPX treatment allows to conclude that positive staining which is indeed related to the presence of polyP bound to the TRPM8 protein. It should be noted that this pool of polyP might have not been extracted using conventional polyP assessment methods. Further, recent reports indicate the existence of polyphosphorylated proteins, suggesting that in addition to the TRPM8 channel, other protein-polyP complexes might be naturally present in mammalian living cells (Azevedo et al. 2015).

6.6 Visualisation and Assessment of PolyP in Living Cells

Approaches described above are very important when polyP can be isolated and needs to be quantified. However, this approach has significant limitations when applied to the eukaryotic cells. In addition to the low levels of polyP, it is present in many different compartments of the cell. For this reason, the extraction of the total

polyP is not very informative as it might include polyP from nucleus, cytoplasm, mitochondria, endoplasmic reticulum, acidocalcisomes and lysosomes. One of the ways around this problem is to use cell fractionation, followed by polyP assays, in each specific fraction (Kumble and Kornberg 1995). Although this method has been used before, it might have significant limitations. For example, in the case of the mitochondrial polyP pool, it is highly variable and depends on the energetic state of the organelle. In fact, energised coupled mitochondria contain higher levels of polyP, compared to the depolarised mitochondria (Pavlov et al. 2010). Thus, assay of polyP in purified mitochondria might not necessarily accurately reflect the levels of polyP in the intact cells, since polyP might be hydrolysed (or accumulated) during the process of mitochondria isolation.

Fluorescent imaging approach appears to be the most direct method for assaying polyP in specific compartments of the living mammalian cells. Unique advantages include the possibility of real-time assays of the levels and location of polyP. At present, the success in this approach has been achieved by the use of DAPI probe. DAPI is generally used to visualise DNA. However, it has been demonstrated that when bound to polyP, the emission spectrum of this probe undergoes long wave shift (Tijssen et al. 1982). This allows to distinguish DAPI-polyP fluorescence. Further, it was demonstrated that the excitation spectrum of DAPI-polyP likewise undergoes long wave shift (Aschar-Sobbi et al. 2008). Thus, the optimisation of both the excitation and the emission wavelengths allowed to increase detection sensitivity of DAPI and used it under conditions of low levels of the polymer, as in case of mammalian cells (Abramov et al. 2007). The most significant limitation of this method is that DAPI-polyP signature signal is not unique and that similar shift is present in DAPI-RNA samples (Martin and Van Mooy 2013) and DAPI-IP6 phosphates (Kolozsvari et al. 2014). Thus, ideally, this protocol needs to be coupled with targeted overexpression of PPX as a control, in order to allow to establish the levels of background fluorescence. This approach was successfully used to evaluate levels of polyP in mitochondria of such cells as living primary neurons (Abramov et al. 2007) and cardiomyocytes (Seidlmayer et al. 2012). Further, DAPI staining protocol has also been adapted to stain polyP in the bone tissue (Omelon et al. 2009). In addition to the selectivity limitation, it should also be mentioned that potentially DAPI can affect cell function. For example, recent studies show that DAPI changes properties of the mitochondrial permeability transition pore, presumably through the interactions with polyP (Elustondo et al. 2015). Alternative to DAPI imaging approach is the use of the recently described fluorescent probe JC-D7/D8 (Angelova et al. 2014). This probe is considerably more selective compared to DAPI and is able to stain living cultured cells. The main limitation of the JC-D7/D8 probe is that it appears to have lower affinity towards polyP compared to DAPI and is highly selective to the ionic strengths of the solution. Thus, depending on the expected levels of polyP in cell, the use of either DAPI or JC-D7/D8 might be preferable. Both of these methods measure levels of free polyP since DAPI and JC-D7/D8 interactions are likely based on high negative charge of polyP. This can be an advantage if the goal is to measure bioavailable levels of polyP and its potential role, for example, as a signalling

molecule (Angelova et al. 2014; Holmstrom et al. 2013; Stotz et al. 2014). However, it should be kept in mind that these probes will not allow to evaluate levels of complexed polyP.

6.7 Future Methods Development

The methods described above have been used in a number of literature reports. While these methods allow to obtain very valuable information regarding the polyP, each of them has specific limitations. Generally, a method needs to be chosen and applied selectively, depending on the experimental question, and in many cases, several methods need to be used in order to obtain conclusive answer. A number of potentially useful methods which will allow more selective and accurate determination of polyP can be developed. One of the promising avenues in the path is the use of polyP-binding domain (PPBD) of the PPX as a detection tool. This peptide can be produced in recombinant form and can highly selectively bind to the polyP. It has been used in experiments with yeast for the immunostaining of the polyP in fixed cells (Saito et al. 2005). Due to its high sensitivity, this method can be adapted to the use in mammalian cells. The limitation of this method is that it requires cell fixation, and during this process, mobile soluble polyP might be lost in washing steps during sample preparation. Another potentially useful approach might involve direct fluorescent labelling of PPBD, followed by endogenous polyP cell labelling. Potentially, PPBD can be also used to create DNA-coding PPBD-FRET protein, which can be transfected into the cells and to produce signal upon binding to the polyP. The major advantage of this approach will include the possibility of its use in living cells for real time and that it is a highly selective assay of polyP levels and localisation.

Conclusions

Detection and quantification of the polyP in higher eukaryotes are a challenging task, due to the presence of the multiple pools of low levels of this polymer. Each of the methods should be selected for using in particular experimental cases, and in many cases, it has to be coupled with other assays to ensure the accuracy and specificity of the measurements. As the interest to the polyP studies grows, new methods are expected to become available in the near future.

References

Abramov AY, Fraley C, Diao CT et al (2007) Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death. Proc Natl Acad Sci U S A 104:18091–18096

Angelova PR, Agrawalla BK, Elustondo PA et al (2014) In situ investigation of mammalian inorganic polyphosphate localization using novel selective fluorescent probes JC-D7 and JC-D8. ACS Chem Biol 9:2101–2110

Aschar-Sobbi R, Abramov AY, Diao C et al (2008) High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. J Fluoresc 18(5):859–866

Ault-Riche D, Fraley CD, Tzeng CM et al (1998) Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. J Bacteriol 180:1841–1847

Azevedo C, Livermore T, Saiardi A (2015) Protein polyphosphorylation of lysine residues by inorganic polyphosphate. Mol Cell 58:71–82

Clark JE, Wood HG (1987) Preparation of standards and determination of sizes of long-chain polyphosphates by gel electrophoresis. Anal Biochem 161:280–290

Clark JE, Beegen H, Wood HG (1986) Isolation of intact chains of polyphosphate from *Propionibacterium shermanii* grown on glucose or lactate. J Bacteriol 168:1212–1219

Elustondo PA, Negoda A, Kane CL et al (2015) Spermine selectively inhibits high-conductance, but not low-conductance calcium-induced permeability transition pore. Biochim Biophys Acta 1847:231–240

Holmstrom KM, Marina N, Baev AY et al (2013) Signalling properties of inorganic polyphosphate in the mammalian brain. Nat Commun 4:1362

Kolozsvari B, Parisi F, Saiardi A (2014) Inositol phosphates induce DAPI fluorescence shift. Biochem J 460:377–385

Kornberg A, Rao NN, Ault-Riche D (1999) Inorganic polyphosphate: a molecule of many functions. Ann Rev Biochem 68:89–125

Kulaev I, Vagabov V, Kulakovskaya T (2004) The biochemistry of inorganic polyphosphates. Wiley, Chichester

Kumble KD, Kornberg A (1995) Inorganic polyphosphate in mammalian cells and tissues. J Biol Chem 270:5818–5822

Lorenz B, Schroder HC (2001) Mammalian intestinal alkaline phosphatase acts as highly active exopolyphosphatase. Biochim Biophys Acta 1547:254–261

Martin P, Van Mooy BA (2013) Fluorometric quantification of polyphosphate in environmental plankton samples: extraction protocols, matrix effects, and nucleic acid interference. Appl Environ Microbiol 79:273–281

Moreno-Sanchez D, Hernandez-Ruiz L, Ruiz FA et al (2012) Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. J Biol Chem 287:28435–28444

Ohtomo R, Sekiguchi Y, Kojima T et al (2008) Different chain length specificity among three polyphosphate quantification methods. Anal Biochem 383:210–216

Omelon S, Georgiou J, Henneman ZJ et al (2009) Control of vertebrate skeletal mineralization by polyphosphates. PLoS One 4:e5634

Pavlov E, Zakharian E, Bladen C et al (2005) A large, voltage-dependent channel, isolated from mitochondria by water-free chloroform extraction. Biophys J 88:2614–2625

Pavlov E, Aschar-Sobbi R, Campanella M et al (2010) Inorganic polyphosphate and energy metabolism in mammalian cells. J Biol Chem 285:9420–9428

Reusch RN (1989) Poly-beta-hydroxybutyrate/calcium polyphosphate complexes in eukaryotic membranes. Proc Soc Exp Biol Med 191:377–381

Reusch RN, Huang R, Kosk-Kosicka D (1997) Novel components and enzymatic activities of the human erythrocyte plasma membrane calcium pump. FEBS Lett 412:592–596

Ruiz FA, Lea CR, Oldfield E et al (2004) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 279:44250–44257

Saito K, Ohtomo R, Kuga-Uetake Y et al (2005) Direct labeling of polyphosphate at the ultrastructural level in *Saccharomyces cerevisiae* by using the affinity of the polyphosphate binding domain of *Escherichia coli* exopolyphosphatase. Appl Environ Microbiol 71:5692–5701

Seidlmayer LK, Gomez-Garcia MR, Blatter LA et al (2012) Inorganic polyphosphate is a potent activator of the mitochondrial permeability transition pore in cardiac myocytes. J Gen Physiol 139:321–331

Sethuraman A, Rao NN, Kornberg A (2001) The endopolyphosphatase gene: essential in *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A 98:8542–8547

Smith SA, Morrissey JH (2007) Sensitive fluorescence detection of polyphosphate in polyacrylamide gels using 4′,6-diamidino-2-phenylindol. Electrophoresis 28:3461–3465

Stotz SC, Scott LO, Drummond-Main C et al (2014) Inorganic polyphosphate regulates neuronal excitability through modulation of voltage-gated channels. Mol Brain 7:42

Tijssen JP, Beekes HW, Van SJ (1982) Localization of polyphosphates in *Saccharomyces fragilis*, as revealed by 4′,6-diamidino-2-phenylindole fluorescence. Biochim Biophys Acta 721:394–398

Werner TP, Amrhein N, Freimoser FM (2007) Specific localization of inorganic polyphosphate (poly P) in fungal cell walls by selective extraction and immunohistochemistry. Fungal Genet Biol 44:845–852

Zakharian E, Thyagarajan B, French RJ et al (2009) Inorganic polyphosphate modulates TRPM8 channels. PLoS One 4:e5404

Inorganic Polyphosphates in the Mitochondria of Mammalian Cells

7

Lea K. Seidlmayer and Elena N. Dedkova

Abstract

This review discusses the current literature on existence and critical roles of the inorganic polyphosphate (polyP) in the mitochondria of mammalian cells. Inorganic polyP is a linear polymer of orthophosphate (P_i) residues linked together by high-energy phosphoanhydride bonds as in ATP. While the length of polyP chain can vary from a few phosphates to several thousands phosphate units long, only short-chain polyPs are detected in mammalian mitochondria. Mitochondrial Ca^{2+} is an essential signaling molecule required for the activation of Ca^{2+}-dependent dehydrogenases and energy production; however, in excess, it could also trigger cell death. PolyP affects mitochondrial Ca^{2+}-transporting systems and mitochondrial metabolism in several ways: (i) it is a potent activator of Ca^{2+}-dependent mitochondrial permeability transition pore (mPTP) and possibly even compose Ca^{2+}-transporting core of the mPTP via formation of the poly-beta-hydroxybutyrate (PHB)-Ca^{2+}-polyP complex in the inner mitochondrial membrane; (ii) reduction of polyP levels increases mitochondrial Ca^{2+}-uptake capacity and decreases the probability of the mPTP opening, and (iii) it is a chelator of Ca^{2+}, among other divalent ions, and therefore it can modify mitochondrial matrix Ca^{2+}-buffering capacity. Furthermore, changes in polyP levels can modulate mitochondrial bioenergetics, generation of the mitochondrial membrane potential, and ATP production by the F_0F_1-ATPase, which can also affect

L.K. Seidlmayer
Department of Internal Medicine 1, University Hospital Wuerzburg,
Oberduerrbacherstr. 6, 97080 Wuerzburg, Germany
e-mail: Seidlmayer_L@ukw.de

E.N. Dedkova, DVM, PhD (✉)
Department of Pharmacology, University of California at Davis,
451 E. Health Sciences Drive, Davis, CA 95616, USA
e-mail: ededkova@ucdavis.edu

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_7

mitochondrial Ca^{2+}-uptake capacity. PolyP concentration is dynamically changed during activation of the mitochondrial respiratory chain and stress conditions such as ischemia-reperfusion and heart failure indicating that polyP is an important component of the normal cell metabolism.

Abbreviations

$[Ca^{2+}]_{em}$	Extramitochondrial Ca^{2+} concentration
$[Ca^{2+}]_{m}$	Mitochondrial Ca^{2+} concentration
Ca^{2+}	Calcium
FCCP	Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
HF	Heart failure
MCU	Mitochondrial Ca^{2+} uniporter
mPTP	Mitochondrial permeability transition pore
PHB	Poly-β-hydroxybutyrate
polyP	Inorganic polyphosphate
PPX	Exopolyphosphatase enzyme
ROS	Reactive oxygen species
TMPD	Tetramethyl-p-phenylenediamine
TMRM	Tetramethylrhodamine, methyl ester

7.1 Introduction

Inorganic polyphosphates (polyPs) are linear polymers of orthophosphate (P_i) residues linked through the common oxygen atoms by high-energy phosphoanhydride bonds similar to adenosine triphosphate (ATP) (Fig. 7.1a) (Kulaev et al. 1999; Kornberg 1999; Rao et al. 2009). At physiological pH, each unit carries a monovalent negative charge which gives polyP a large capacity for cation binding and exchange (Reusch 2000). PolyP chain length can vary from just a few phosphates to several thousands phosphate units long, depending on the organism and the tissue in which it is synthesized (Kornberg 1995, 1999; Dedkova and Blatter 2014; Dedkova 2016). Based on the length of polyP chain, these polymers are classified in three different categories – (i) very long-chain polyPs, ranging from hundreds to thousands of P_i units, existing mainly in microorganisms (Kulaev and Vagabov 1983), (ii) medium-chain polyPs (composed by 60–100 phosphate residues) (Kumble and Kornberg 1995), and (iii) short-chain polyPs (composed by 3–60 phosphate residues) (Seidlmayer et al. 2012b; Dedkova and Blatter 2014; Kumble and Kornberg 1995) – that are abundant in mammalians.

The discovery of polyPs goes back to the beginning of the nineteenth century when Graham obtained a vitreous phosphate by fusing NaH_2PO_4 at 700–800 °C for several hours followed by rapid cooling (Graham's salt) (Graham 1833). Therefore, it is not surprising that polyP was found in volcanic condensates and deep oceanic

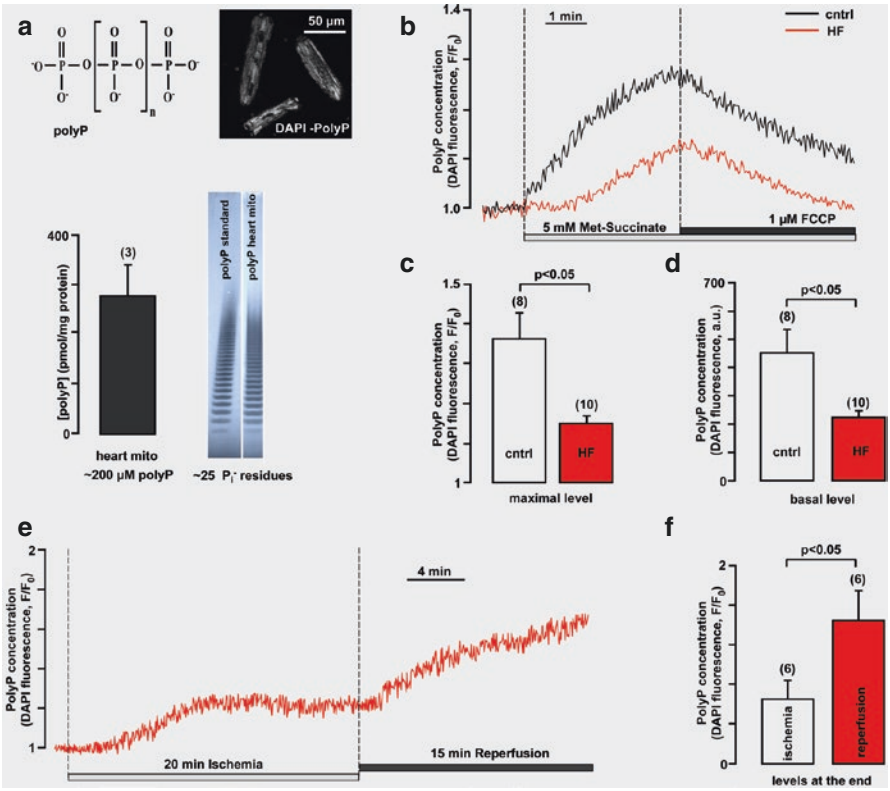

Fig. 7.1 Mitochondrial polyP detection in healthy and heart failure cardiac myocytes. (**a**) The *upper* panel shows the structure of inorganic polyphosphate. The n represents the number of phosphate residues in the polyphosphate chain. It could vary from ten to hundreds of units. The *right* panel image demonstrates polyP detection in freshly isolated rabbit ventricular myocytes using DAPI as a sensor for polyP (λ_{ex} = 408 nm, λ_{em} = 552–617 nm). The *bottom* panel shows the average amount of polyP in rabbit heart mitochondria (*eft*) and gel images of polyP standard and polyP sample from isolated rabbit mitochondria (*right*). (**b**) Original recordings of DAPI fluorescence changes in intact cardiac myocytes stimulated with 5 mM methyl succinate followed by 5 μM FCCP from control (*black*) and failing myocytes (*red*). DAPI fluorescence represents changes in polyP concentration. (**c**) Average values of maximal DAPI fluorescence after methyl-succinate addition in control (*black*) and heart failure (*red*) cells. (**d**) Average values of basal DAPI fluorescence in control (*black*) and heart failure (*red*) myocytes. (**e**) Original recordings of DAPI fluorescence changes in intact cardiac myocytes exposed to 20 min of simulated chemical ischemia followed by 15 min of reperfusion period in normal Tyrode solution. (**f**) Average values of maximal DAPI fluorescence at the end of 20 min ischemia and 15 min reperfusion in control rabbit ventricular cells (Modified with permission from Seidlmayer et al. 2012a, b, 2015)

steam vents possibly even preceding life on the planet Earth (Brown and Kornberg 2004). Professor Igor S. Kulaev in the Russian Academy of Sciences has pioneered the field of polyP studies (Belozerskii and Kulaev 1957; Kulaev and Belozerskii 1957; Kulaev et al. 1960) and explored the widespread occurrence of polyP, methodologies for its detection, biosynthetic pathways (Nesmeianova et al. 1973, 1974;

Kulaev and Bobyk 1971) in prokaryotes, and unicellular eukaryotes. His group discovered that prokaryotes and unicellular eukaryotes (and possibly all early living organisms on Earth) used polyP as long-term sources of phosphate (P_i) and energy (Kulaev and Vagabov 1983). It is possible that polyP has allowed microorganisms to survive Earth's extreme primitive environment in the past. Kulaev's group was the first to demonstrate that intestinal alkaline phosphatase is involved in polyP synthesis and identified that genes phoR and phoS are involved in the formation of a repressor complex for both alkaline phosphatase and polyphosphatase (Maraeva et al. 1979).

The torch of polyP studies has been picked up by Arthur Kornberg at Stanford University who won the Nobel Prize in Physiology or Medicine in 1959 for the discovery of the deoxyribonucleic acid (DNA) polymerase in the intestinal bacterium *Escherichia coli* (*E. coli*). Kornberg and his group (Kumble and Kornberg 1996; Kumble et al. 1996; Wurst and Kornberg 1994; Wurst et al. 1995) were able to discover the enzymes for the synthesis and utilization of polyP and genetically modify them (Crooke et al. 1994) to obtain the clues for new metabolic functions of polyP in bacteria, yeast, and animal cells. Specifically, Kornberg's group was first to purify the polyphosphate kinase 1 (PPK1) of *E. coli* (Ahn and Kornberg 1990), the main enzyme which catalyzes the reaction of reverse transfer of energy-rich phosphate residues from ATP to polyP and from polyP to ADP, linking energy-rich pools.

In collaboration with Dr. Rosetta Reusch from Michigan State University, Kornberg recognized the role of polyP in bacterial transformation, the mechanism by which inclusion of polyP in a membrane complex with poly-β-hydroxybutyrate (PHB) and Ca^{2+} enables the cell to become competent to take up DNA and make them genetically transformed (Castuma et al. 1995; Huang and Reusch 1995). Furthermore, Kornberg's group identified the basis for polyP regulation in the bacterial responses to physical and chemical stress and adjustments for survival in the stationary phase of culture growth and development (Kim et al. 1998, 2002; Ault-Riche et al. 1998). The authors demonstrated that polyPs are required for motility of bacterial pathogens (Rashid et al. 2000a; Rashid and Kornberg 2000). Knocking down PPK1 significantly impaired swimming motility, biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa* (Rashid et al. 2000b). Since the motility of pathogens is essential to invade and establish systemic infections in host cells, this impairment in motility suggested a crucial and essential role of polyP in bacterial pathogenesis. The conservation of PPK1 among many bacterial pathogens and its absence in eukaryotes suggest that PPK1 might be an attractive target for antimicrobial drugs.

Furthermore, Kumble and Kornberg (1995) screened a large number of mammalian cells and tissues to confirm the presence of polyP in the rodent liver, kidney, lungs, brain, and heart (Kumble and Kornberg 1995). Since then, the presence of polyP has been demonstrated in a variety of the mammalian cells and tissues including humans where, depending on location, polyP plays different biological roles (Wood and Clark 1988; Kulaev et al. 1999; Kornberg et al. 1999; Dedkova and Blatter 2014; Dedkova 2016). However, in a striking contrast to microorganisms

where polyPs are present in millimolar (50–120 mM) concentrations, levels of 25–200 μM (in terms of P_i residues) were detected in mammalian tissues with different distributions in subcellular organelles (Kumble and Kornberg 1995; Seidlmayer et al. 2012b; Dedkova and Blatter 2014; Dedkova 2016). The exceptions are platelets and mast cells which contain millimolar concentrations of polyP in electron-dense granules (Ruiz et al. 2004). These electron-dense granules have similarities to the volutin granules that are widely distributed in bacterial cells and parasites (Docampo et al. 2005). These electron-dense acidic organelles are rich in calcium and polyphosphate and therefore were called acidocalcisomes. Acidocalcisomes represent the only organelles that have been conserved during evolution from prokaryotes to eukaryotes. Acidocalcisomes have been linked with several functions, including storage of cations and phosphorus, polyP metabolism, calcium homeostasis, maintenance of intracellular pH homeostasis, and osmoregulation. Most recent data from our laboratory (Demirkhanyan et al. 2016; Dedkova 2016) indicate that failing cardiac myocytes accumulate polyP in the granule-like structures which overlapped with poly-β-hydroxybutyrate (PHB), a polymerized form of the ketone body β–hydroxybutyrate. The exact function of these polyP/PHB granules is unknown, and they induce a significant interest in the scientific community. In this review, we will primarily focus on the role of polyphosphates in the mitochondrial function of mammalian cells.

7.2 From Industry and Agriculture to Medicine: Sources of PolyPs in Mammalian Cells

PolyPs are present and detected in human cells (Pisoni and Lindley 1992; Cowling and Birnboim 1994; Lorenz et al. 1997; Abramov et al. 2007), despite the fact that no enzyme responsible for polyP generation in mammalian cells has been identified (Dedkova and Blatter 2014; Dedkova 2016). PolyPs, however, have been essential components of our diets, since they extensively used as food preservatives and as components of the commercial water-purifying systems (Kulakovskaya et al. 2012). PolyPs can regulate water softness via process called sequestration, i.e., via formation of soluble Ca^{2+} and magnesium (Mg^{2+}) polyP complexes. By forming soluble complexes with Ca^{2+}, polyP is very effective against Ca^{2+} carbonate scale deposits in the water system. Besides Ca^{2+} and Mg^{2+}, PolyPs are able to bind and sequester soluble iron, preventing occurrence of rusty water and corrosion of the water pipes. The sequestering process minimizes the risk of discoloration, staining, and scaling, decreases chlorine demand, and improves the taste of drinking water. PolyP water filter cartridges are commercially available and recommended for use with ice machines, coffee and vending machines, food service equipment, water heaters, air conditioning equipment, and many other types of water processing equipment to inhibit scale and rust buildup.

PolyPs are also used as efficient soil and aquaculture fertilizers due to their low cost, nontoxicity, and biodegradability. Ammonium polyphosphate, an inorganic salt of polyphosphoric acid and ammonia, is one of the frequently used

phosphoric fertilizers (Vasant et al. 2009; Kulakovskaya et al. 2012). It is applied both in soil and in aquaculture supplement microalga fertilization, which is used as a feed for farmed fish. PolyP fertilizers are more effective than rock phosphate (apatite) and superphosphates (a mixture of dicalcium phosphate and calcium sulfate). Commercially available ammonium polyphosphates are mixtures of ortho- and polyphosphates with long polymer chains (>1,000). PolyP fertilizers have to breakdown to the orthophosphate (P_i) form in order to be absorbed by plants.

PolyPs are legally permitted food additives (E450–452) (Sen et al. 2005; EEFS 2013) and are widely used to treat fish, fish fillet, shrimp, and meat products to improve their water-holding capacity, reduce the amount of thaw drip, and increase their freezing capacity. Due to unique ability of polyP to bind cations, buffer pH, and retain water, polyP helps to maintain an optimal pH level in food and protects food against undesirable change of product color during storage. The antibacterial effect of polyP helps to extend the product shelf life.

When consumed with food, polyPs are broken down to single phosphate units in the stomach: polyP is easily degraded to orthophosphate by the alkaline phosphatase of the intestines and by other enzymes of human organism (Lorenz and Schroder 2001). However, human gastrointestinal tract bacteria (probiotics) produce polyPs. In fact, polyP is responsible for probiotic actions that protect the intestinal epithelia from oxidant stress and improve epithelial injury due to excess inflammation (Segawa et al. 2011). Furthermore, probiotic administration attenuates myocardial infarction following ischemia-reperfusion injury (Lam et al. 2012; McCafferty et al. 2012) and heart failure following myocardial infarction (Gan et al. 2014) in the rat. PolyP levels were not assessed in these studies; however, it is plausible to speculate that polyP produced by gut microbiota could also exert a cardioprotective role for the host organism.

In the other side, excessive consumption of polyPs and phosphates are associated with the development of nephrocalcinosis (a deposition of calcium salt in the renal parenchyma due to hyperparathyroidism) (Matsuzaki et al. 2001), increased bone resorption (Takeda et al. 2014), endothelial dysfunction (Watari et al. 2015), and increased lung tumorigenesis (Jin et al. 2009). Since both phosphate and polyPs are important participants of metabolic and signaling processes (see below), their consumption needs to be controlled, and their effects on human health need to be studied in more details.

7.3 Pathways for PolyP Synthesis and Degradation

The synthesis of polyP in prokaryotes is primarily mediated by the polyphosphate kinase (PPK1) which catalyzes the transfer of P_i from ATP to polyP (Kornberg 1957) in the following reaction.

$$\mathbf{PolyP_n} \leftrightarrows + \mathbf{ATP PolyP_{n+1}} + \mathbf{ADP}$$

PPK1 also catalyzes polyP-driven ATP synthesis by its reverse reaction. PPK1 is encoded by *ppk1* gene with a well-known sequence (Ahn and Kornberg 1990; Akiyama et al. 1992) that opens unique opportunities for genetic manipulation. In fact, using the *ppk1*-lacking null mutant of *Pseudomonas aeruginosa and E. coli*, it was discovered (Ishige et al. 2002; Zhang et al. 2002) that bacterial cells contain another enzyme, PPK2 (Sureka et al. 2009; Ishige et al. 2002) which mediates polyP-driven formation of Guanosine-5′-trisphosphate (GTP), a molecule known to have important roles in cell signaling as well as DNA, RNA, protein, and polysaccharide synthesis (Chakrabarty 1998). In contrast to PPK1, PPK2 preferentially catalyzes the reverse reaction.

Cells from higher-order organisms may use a different mechanism for polyP generation since no *ppk* gene homologues have been found in mammals although PPK1 is structurally similar to phospholipase D (Zhu et al. 2005). In respect to low-order eukaryotes, *ppk1* homologues have been found in *Dictyostelium discoideum* (Zhang et al. 2005, 2007). The slime mold *D. discoideum* retains polyphosphate kinase activity due to the presence of a bacterial *ppk1* homologue, *DdPPK1*. The enzyme responsible for sustaining the polyP levels, DdPPK2, is an actin-related protein (Arp) complex, which is polymerized into an actin-like filament concurrently with the reversible synthesis of polyP from ATP (Gomez-Garcia and Kornberg 2004; Zhang et al. 2007; Rao et al. 2009). Screening of the human protein BLAST database using the proteins that retain DdPPK2 activity in *D. discoideum* (ERpA ArpD and ACT28) revealed highly significant matches of actin-like proteins. The occurrence of polyP synthesizing activity in these proteins needs to be verified (Hooley et al. 2008).

Furthermore, plasma membrane Ca^{2+} ATPase from human erythrocytes can function as a polyphosphate kinase, i.e., it exhibits ATP-polyphosphate transferase and polyphosphate-ADP transferase activities (Reusch et al. 1997). The potential role of mitochondrial F_1F_0-ATP synthase in polyP generation has been proposed (Pavlov et al. 2010; Seidlmayer et al. 2012a) and will be discussed below.

The degradation of polyP is catalyzed by several endopolyphosphatases (PPNs) (Kumble and Kornberg 1996) and exopolyphosphatases (PPXs) (Akiyama et al. 1993; Lorenz et al. 1994; Andreeva and Okorokov 1993; Wurst and Kornberg 1994; Wurst et al. 1995). In mammalians, a human metastasis regulator protein H-prune was identified as a short-chain-specific exopolyphosphatase (Tammenkoski et al. 2008). It is speculated that H-prune protein could be the missing exopolyphosphatase in animals since it exhibits ~91 % homology with the sequences of yeast exopolyphosphatase PPX1 (Tammenkoski et al. 2008). This discovery also suggests that metastatic effects of H-prune in cancer cells are mediated by polyP (Tammenkoski et al. 2008). Furthermore, a long-chain endopolyphosphatase was purified from rat and bovine brain (Kumble and Kornberg 1996), and mammalian intestinal alkaline phosphatase was characterized as a very active exopolyphosphatase (Lorenz and Schroder 2001). BLAST search database revealed ~41 % homology between yeast exopolyphosphatase PPN1 gene and human acid sphingomyelinase-like phosphodiesterase gene (Duan 2006; Kulakovskaya and Kulaev 2013). Sphingomyelinase-like phosphodiesterase plays antiproliferative and anti-inflammatory roles in the colon via ceramide generation, reducing the

lysophosphatidic acid formation and inactivating the platelet-activating factor (Duan 2006). Mammalian thymidylate kinase actually resembles bacterial PPK2 activity (Whitehead et al. 2013, 2014).

Additionally, human mitochondrial NAD kinase utilizes not only ATP but also polyP as the phosphoryl donor (Ohashi et al. 2012). NAD kinase is the sole $NADP^+$-biosynthetic enzyme known to catalyze phosphorylation of NAD^+ to yield $NADP^+$ and plays a role in the defense against mitochondrial oxidative stress (Pollak et al. 2007).

Thus, it seems that at least some of the gene-encoding polyphosphate-metabolizing enzymes in the lower eukaryotes have been retained in mammalian cells as well.

7.4 Mitochondrial Metabolism of PolyP in Mammalian Cells

Lynn and Brown (1963) were first to report polyP formation by rat liver mitochondria; however, their data were not very convincing due to difficulties to detect small amounts of polyP with techniques available at that time. Kumble and Kornberg (1995) confirmed this fact and determined that mitochondria of rat liver contain ~11 μM of polyP, the amount which is significantly smaller compared to polyP detected in nuclei (~89 μM) and plasma membrane (~43 μM). We (Seidlmayer et al. 2012b) detected that mitochondria from rabbit hearts contain ~200 μM (280 pmol/mg of protein) short-chain polyP with an average chain length of 25 orthophosphates (Fig. 7.1a).

Given the physical and chemical properties of polyP, polyPs located inside mitochondria can regulate mitochondrial function in several ways. First, polyP as a high-energy polymer can serve as a membrane-potential-independent source of ATP production and thus affect the activity of the ATP synthase and the respiratory chain. Second, polyPs have strong ability to chelate divalent cations and, therefore, could affect mitochondrial Ca^{2+}-buffering capacity and alter the Ca^{2+}-dependent regulation of multiple mitochondrial enzymes. Third, as a highly charged polyanion, polyP could directly bind to mitochondrial proteins and modify their activities.

As we discussed above (see Sect. 7.3), currently no enzymes responsible for polyP generation in mammalian cells have been identified, purified, and cloned. Several labs, however, demonstrated that polyP generation in mammalian cells depends on the metabolic state of the mitochondria (Pavlov et al. 2010; Seidlmayer et al. 2012a, b). Levels of polyP in isolated rat liver mitochondria, cultured intact cells (astrocytes, HEK 293), and rabbit cardiomyocytes were increased by substrates of the mitochondrial respiratory chain and in turn reduced by the mitochondrial complex I inhibitor rotenone or respiratory chain uncoupler carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Cell treatment with oligomycin, an inhibitor of mitochondrial F_1F_0-ATP synthase, inhibited production of polyP suggesting that in mammalian mitochondrial polyP production is closely related to the activity of the oligomycin-dependent F_1F_0-ATP synthase. However, the validation whether or not F_1F_0-ATP synthase is polyP-generating enzyme is still pending.

Furthermore, we found that mitochondrial polyP metabolism (Seidlmayer et al. 2012a) is significantly impaired in intact ventricular myocytes isolated from animals with heart failure (HF, combined aortic insufficiency, and stenosis model) (Dedkova et al. 2013) compared to healthy control rabbits. Levels of polyP were monitored using the fluorescent probe DAPI (Fig. 7.1a), with a protocol optimized specifically for polyP detection (Aschar-Sobbi et al. 2008; Seidlmayer et al. 2012a, b). Activation of the mitochondrial complex II with membrane-permeable substrate methyl succinate led to the increase in DAPI fluorescence by 36 ± 8 % ($n = 8$) in control cardiac myocytes, indicating significant stimulation of the mitochondrial polyP production (Fig. 7.1b, c). When mitochondrial respiratory chain was uncoupled with FCCP, DAPI fluorescence decreased by 29 ± 4 % ($n = 8$) presumably due to the stimulation of polyP hydrolysis. These data demonstrate that the polyP levels in cardiac myocytes depend on the energetic status of the cell and the degree of coupling of the mitochondrial respiratory chain. In HF myocytes, however, addition of methyl succinate induced only a moderate increase in DAPI fluorescence (16 ± 2 %, $n = 10$) (Fig. 7.1b, c) (Seidlmayer et al. 2012a). Compared to control cells, basal levels of polyP were significantly lower in conditions of HF (224 ± 21 a.u. in HF versus 453 ± 80 in control) (Fig. 7.1d) (Dedkova and Blatter 2014). Furthermore, polyP levels inside mitochondria of intact ventricular myocytes were increased during cell exposure to the simulated ischemia-reperfusion (Fig. 7.1e, f). These conditions of simulated ischemia-reperfusion were accompanied by a significant increase in reactive oxygen species (ROS) generation (Seidlmayer et al. 2015) raising the intriguing possibility that similarly to bacteria, mammalian cells could produce polyP in response to cellular stress. Impaired polyP synthesis observed in HF myocytes could result from the complex remodeling processes during cardiac hypertrophy and heart failure. Studies performed in bacterial cells indicate that polyP acts as an efficient protein chaperon which stabilizes proteins in vivo, diminishes the need for other chaperone systems to survive proteotoxic stress (temperature, low pH, oxidants) conditions, and protects a wide variety of proteins against stress-induced unfolding and aggregation (Gray et al. 2014; Kampinga 2014). Particularly, it was shown that wild-type *E. coli* strains generated significant amounts of polyP in response to oxidative stress. The discovery that polyP has stress-protective chaperone activities that resemble the activity of small heat shock proteins is very intriguing. More studies are required to determine protein targets of polyP in mammalian cells and exact mechanisms for protein aggregation prevention by polyP.

7.5 Effects of PolyP Depletion on the Respiratory Chain Activity

The chemical and physical properties of polyP, including its high negative charge, its ability to form complexes with Ca^{2+}, and form high-energy bonds, underlie its potential to play an important role in cell metabolism. A comprehensive study from Dr. Pavlov's lab (Abramov et al. 2007) evaluated the effects of a reduction

in mitochondrial polyP content in several cell culture lines (HEK 293, HepG2, and C2C12) on the mitochondrial function. PolyP depletion was achieved by overexpression of a DNA construct encoding a fusion protein, MGP, composed of mitochondrially targeted GFP (MTS-GFP) and an exopolyphosphatase enzyme from yeast (scPPX1) that specifically hydrolyzes polyP into inorganic phosphate (Abramov et al. 2007). Reduction of polyP levels in mitochondria of living cells was confirmed by a decrease in DAPI-polyP fluorescence levels, and then several parameters of mitochondrial function (mitochondrial membrane potential and the redox state of NADH) were monitored in control and polyP-depleted cells. The authors found that in cells with overexpressed scPPX1 (i.e., with depleted polyP levels), mitochondrial membrane potential ($\Delta\Psi_m$) was reduced up to 40–50 %. At the same time, the reduction of polyP levels by scPPX1 expression was associated with an increase in NADH autofluorescence suggesting an increase in the reduced state of NADH, the main substrate of the respiratory chain. Typically, a decrease in $\Delta\Psi_m$ may result from an increased leak, decreased substrate supply, or decreased respiratory activity. The authors (Abramov et al. 2007) concluded that polyP depletion most likely decreased $\Delta\Psi_m$ by inhibition of mitochondrial respiration, suggesting that polyP plays a role in the maintenance of normal mitochondrial respiration. To verify this hypothesis, the activity of the mitochondrial respiratory chain was monitored indirectly by measuring changes in NADH autofluorescence during supplementation of substrates for the mitochondrial complexes I, II, and IV. The level of NADH autofluorescence could change dynamically depending on the mitochondrial matrix environment: increased leak at the respiratory chain would shift NADH fluorescence into more oxidized state (i.e., lead to the decrease in NADH fluorescence), whereas impaired respiration would shift NADH into more reduced state (i.e., lead to the increase in fluorescence). They found that the application of complex I substrates (5 mM of pyruvate and 5 mM glutamate) induced a similar increase in NADH autofluorescence levels in both control and polyP-depleted cells; however, this increase translated in much smaller (about 27 %) increase in the mitochondrial membrane potential in polyP-depleted cells suggesting that the activity of the complex I was significantly impaired in polyP-depleted cells. Similar results were obtained when mitochondrial complex II activity was stimulated with the membrane-permeable analog of a complex II substrate, methyl succinate (5 mM). Methyl succinate restored the mitochondrial membrane potential in rotenone-treated control and polyP-depleted cells, confirming the activity of complex II after polyP depletion. However, similarly to the complex I substrates, the effect of methyl succinate was 36.8 ± 3.1 % smaller in polyP-depleted compared to control cells. When complex IV substrates, 200 μM tetramethyl-p-phenylenediamine (TMPD) in combination with 5 mM ascorbate, were applied, both control and polyP-depleted cells responded with an increase in $\Delta\Psi_m$; however, again the increase in TMRM fluorescence in polyP-depleted cells was ~33 % smaller compared to the control. Altogether, these data demonstrate that under conditions of polyP depletion, the activity of three major respiratory complexes is impaired suggesting the critical role of polyP in respiratory chain regulation.

7.6 Role of the Inorganic PolyP in Mitochondrial Ca^{2+} Uptake and Ca^{2+} Buffering

Mitochondrial Ca^{2+} uptake is an essential stimulator of the mitochondrial energy production to meet cellular demand, but in excess, it could also trigger cell death. Due to unique ability of polyP to bind divalent ions (see above), it is expected that polyP presence in mitochondria would provide binding sites for Ca^{2+} and therefore contribute to Ca^{2+} buffering within mitochondria. To explore this possibility, the effect of polyP depletion in mitochondria was monitored in non-excitable cells (Abramov et al. 2007) and cardiac ventricular myocytes (Seidlmayer et al. 2012b). PolyP depletion was achieved by overexpression of a DNA construct encoding a fusion protein, MGP, composed of mitochondrial-targeting sequence GFP (MTS-GFP) and an exopolyphosphatase enzyme from yeast (scPPX1) that specifically hydrolyzes polyP into inorganic phosphate (Fig. 7.2a). Mitochondrial location of the GFP-PPX construct was confirmed by colocalization with the mitochondrial membrane-potential-sensitive dye TMRM (Fig. 7.2a, upper panel). Similar to the non-excitable cells, polyP depletion in cardiac myocytes was confirmed by the decrease in DAPI fluorescence (see Fig. 7.2a, low panel and DAPI spectrum on the right in Fig. 7.2b). In both cases, cells were permeabilized with low concentrations of digitonin, which selectively permeabilizes the plasma membrane, but not the inner mitochondrial membrane (Dedkova and Blatter 2012), and then cells were exposed to the different extramitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_{em}$). Mitochondrial matrix Ca^{2+} concentration ($[Ca^{2+}]_m$) was monitored with the high-affinity fluorescent dyes Rhod-2 (Seidlmayer et al. 2012b) or X-Rhod-1 (Abramov et al. 2007; Seidlmayer et al. 2012b) accumulated within mitochondrial matrix. In non-excitable cells, it was demonstrated that cell exposure to 1 μM of $[Ca^{2+}]_{em}$ did not produce any significant changes in both control and polyP-depleted cells; however, addition of 20 μM Ca^{2+} clearly increased $[Ca^{2+}]_m$ in polyP-depleted cells while it led to a decrease in X-Rhod-1 fluorescence in control cells suggesting a release of Ca^{2+} and/or fluorescent dye from mitochondria (Abramov et al. 2007). This decrease of X-Rhod-1 fluorescence was prevented by cell preincubation with 0.5 μM cyclosporin A (CsA), which desensitizes the mitochondrial permeability transition pore (mPTP), a nonspecific large pore in the inner mitochondrial membrane. This data suggest a possible involvement of polyP in mPTP activation and possibly formation (which will be discussed below and reviewed in Seidlmayer et al. (2012a, b) and Dedkova and Blatter (2014). The authors obtained similar results when they utilized a low-affinity Ca^{2+} indicator, Rhod-5N. In this case, addition of 1.2 mM Ca^{2+} induced a fast loss of Rhod-5N fluorescence from control mitochondria; however, a further increase in Rhod-5N fluorescence was observed in PPX overexpressing (i.e., polyP-depleted cells). Subsequent addition of the mitochondrial uncoupler FCCP led to the release of Ca^{2+} from the mitochondrial matrix in polyP-depleted cells, suggesting that PPX overexpression allowed mitochondria to accumulate and store substantial additional Ca^{2+} without promoting mPTP opening. Surprisingly, the increased accumulation of Ca^{2+} in polyP-depleted cells was observed under conditions of the decreased mitochondrial membrane potential (Abramov et al. 2007).

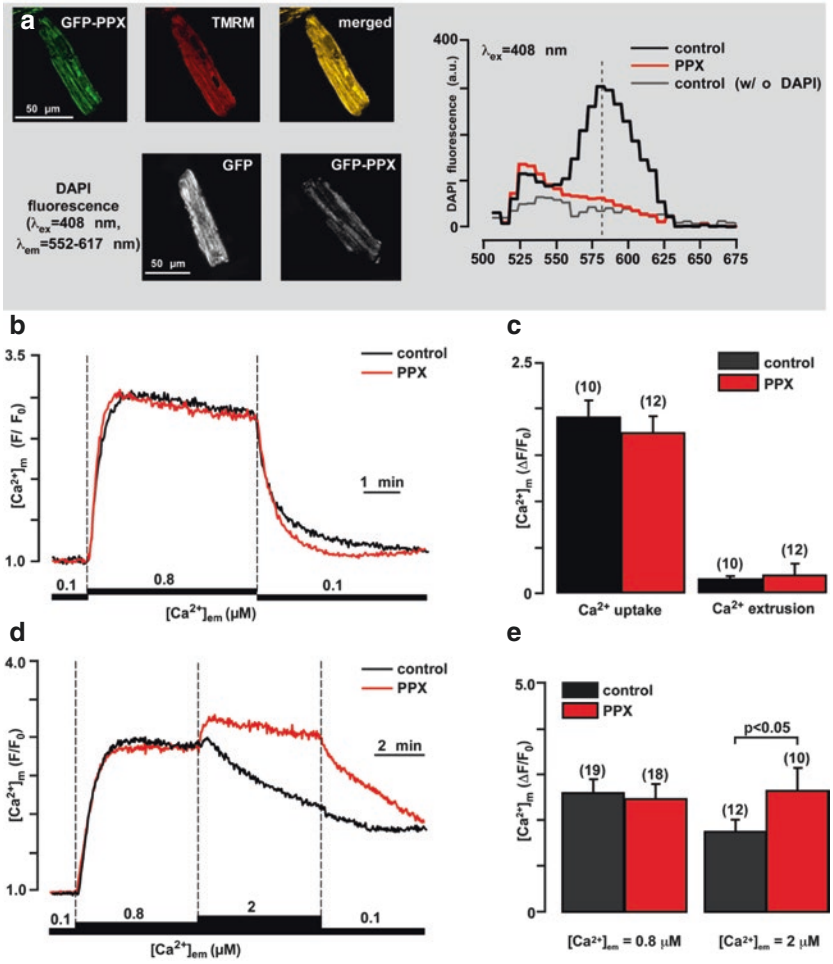

Fig. 7.2 PolyP depletion in mitochondria of cardiac myocytes prevents mitochondrial Ca^{2+} release induced by high Ca^{2+}. (**a**) Images of control GFP (*left*) and PPX-expressing cardiac myocytes (*right*). The *upper* panel shows global GFP fluorescence at 500–530 nm that reveals the mitochondrial fluorescence pattern in PPX-expressing cells and a homogeneously distributed fluorescence in control cells. The *upper* right panel shows colocalization of GFP-PPX signal with mitochondria. TMRM was used as a mitochondrial signal, and the degree of overlay is presented in shades of *yellow* in the merged image. The *bottom* panel shows the decrease in DAPI fluorescence in polyP-depleted cells. (**b**) Fluorescence spectrum of DAPI (5 µM) loaded myocytes expressing control GFP (*black*), PPX (*red*), and control GFP cells not loaded with DAPI (*gray*). (**c**) Original recordings of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) in permeabilized X-Rhod-1-loaded cells upon elevation of $[Ca^{2+}]_{em}$ from 0.1 to 0.8 µM and subsequent return to 0.1 µM in control (*black*) an polyP-depleted (*red*) cells. (**d**) Average values of mitochondrial Ca^{2+} uptake (*left*) and extrusion (*right*) measured at 5 min of Ca^{2+} addition and removal in control and polyP-depleted (PPX) cells. (**e**) Original recordings of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$ in permeabilized X-Rhod-1-loaded cells upon stepwise elevation of $[Ca^{2+}]_{em}$ from 0.1 to 0.8 to 2 µM and subsequent return to 0.1 µM in control (*black*) an polyP-depleted (*red*) cells. (**f**) Average values for $[Ca^{2+}]_m$ amplitude measured at 5 min of 0.8 (*left*) and 2 µM (*right*) Ca^{2+} exposure (Modified with permission from Seidlmayer et al. 2012b)

Since Ca^{2+} uptake is potential dependent, it would be expected to observe a decreased mitochondrial Ca^{2+} uptake in polyP-depleted cells. In general, these results suggest that polyP can buffer free Ca^{2+} inside of the mitochondrial matrix of the non-excitable cells and contribute toward activation of the mPTP. In a different study, Solesio et al. compared $[Ca^{2+}]_m$ levels during mitochondrial Ca^{2+} overload in cultured differentiated and undifferentiated hepatoma cells (HepG2) induced by cell exposure to Ca^{2+} ionophore ionomycin (Solesio et al. 2016). The authors found that ionomycin application to undifferentiated cells induced a higher rise in free $[Ca^{2+}]_m$ which correlated with higher polyP levels in these cells compared to the differentiated cells. Furthermore, when polyP levels were decreased by overexpression of the mitochondrially targeted scPPX1, ionomycin-induced $[Ca^{2+}]_m$ response in undifferentiated cells was significantly reduced. These data indicate that $[Ca^{2+}]_m$ levels during mitochondrial Ca^{2+} overload were lower when polyP levels were lower or reduced which could be explained by the ability of polyP to regulate phosphate-Ca^{2+} interaction.

We obtained similar results in permeabilized rabbit cardiac myocytes which were exposed to low (0.8 μM) and high (2 μM and above) $[Ca^{2+}]_{em}$. In contrast to non-excitable cells (Abramov et al. 2007), polyP depletion did not affect the ability of mitochondria to accumulate Ca^{2+} significantly, at least within the range of $[Ca^{2+}]_{em}$ used in the study. In our study (Seidlmayer et al. 2012b), application of 0.8 μM $[Ca^{2+}]_{em}$ initiated a mitochondrial Ca^{2+} uptake in both control and polyP-depleted cells with essentially no difference observed in the amplitude, rate of Ca^{2+} accumulation, and Ca^{2+} release (Fig. 7.2c, d). However, when higher Ca^{2+} concentrations were applied, $[Ca^{2+}]_{em}$ continued to raise in polyP-depleted cells while fluorescence of X-Rhod-1 declined in control cells in CsA-sensitive manner (Fig. 7.2e, f). These data point against a significant contribution of the mitochondrial polyP toward Ca^{2+} buffering in the mitochondrial matrix of cardiac myocytes at least upon mitochondrial uptake stimulated by Ca^{2+} in the range of 0.8–2 μM. This could be explained by the fact that mitochondria of cardiac myocytes contain only 200 μM polyP (see Fig. 7.1a above) versus millimolar amounts found in bacteria which would support a regulatory role for mitochondrial Ca^{2+} uptake and release rather than provide a substantial Ca^{2+}-buffering capacity. It is possible that polyP depletion can increase concentration of the inorganic phosphate inside mitochondria which is known to modulate mitochondrial Ca^{2+} uptake (Sedova et al. 2006) and Ca^{2+}-buffering capacity (Wei et al. 2012). However, millimolar concentrations of phosphate are required to affect mitochondrial Ca^{2+} uptake significantly (Sedova et al. 2006; Wei et al. 2012). Two distinct modes of the mitochondrial Ca^{2+} uptake via mitochondrial Ca^{2+} uniporter (MCU), "MCU_{mode1}" and "MCU_{mode2}," are described in cardiac mitochondria (Wei et al. 2012). Mode 1 has a relatively low capacity, is engaged by small (0.1–2 μM) increases in $[Ca^{2+}]_{em}$, is completely inhibited by only relatively high levels (~1 μM) of the MCU inhibitor Ru360, and triggers relatively large changes in matrix-free calcium. In contrast, mode 2 is responsible for the bulk of calcium transport for external calcium in the range of 2–10 μM, is inhibited by 0.1 μM Ru360, and is associated with very small changes in matrix-free calcium owing to very high Ca^{2+} buffering. The data and analyses presented by the authors support the conclusion that mode 1 Ca^{2+} transport functions as a signal, perhaps to dynamically

modulate oxidative phosphorylation, whereas mode 2 is associated with a high-capacity Ca^{2+}-buffering function. The study of Wei et al. (2012) also suggested that the trigger for activation of the mitochondrial permeability transition pore (mPTP) is unlikely to be free matrix Ca^{2+} itself but could be a downstream byproduct of total mitochondrial Ca^{2+} loading and possibly polyP-Ca^{2+} complex formation (see below).

7.7 Inorganic PolyP and Activation of the Mitochondrial Permeability Transition Pore (mPTP)

The data described above suggest the involvement of polyP in either direct activation/formation of the mPTP or at least mediation of Ca^{2+} transport via mPTP based on the CsA sensitivity of Ca^{2+} release component (see Sect. 7.6). In 1988 Reusch and Sadoff extracted a complex from plasma membrane of *E. coli* consisting of poly-β-hydroxybutyrate (PHB)/polyP/Ca^{2+} with molar ratios approximating 1:1:0.5 (Reusch and Sadoff 1988). The estimated chain length of PHB was about 120–200 subunits, and the polyP chain was estimated to be about 130–170 subunits. The extracted complex, when incorporated into liposomes, formed a channel which exhibited a lipid phase transition in the same temperature range as that of the membrane complex in whole cells as well as the same properties of irreversibility, lability, and sensitivity to chelating buffers (Reusch and Sadoff 1988). It was proposed that the PHB forms an exolipophilic-endopolarophilic helix around an inner framework helix of calcium polyphosphate. The Ca^{2+} ions link the two polymers by forming ionic bonds with phosphoryl oxygens of the polyphosphate and ion-dipole bonds with the ester carbonyl oxygen of the PHB. It was suggested that this symmetrical structure forms a channel through the membrane and may play a role in the transport of Ca^{2+}, phosphate, and DNA. Indeed, by using bilayer techniques, it was confirmed that the reconstitution of the PHB/polyP/Ca^{2+} complex from *E. coli* into planar lipid bilayers yields 100 pS cationic-selective channels with a preference for Ca^{2+} ions (Das et al. 1997). These data were confirmed by another group (Pavlov et al. 2005a), who reported that PHB/polyP/Ca^{2+} complex from *E. coli* can also form larger, weakly selective pores, with a maximal conductance ranging from 250 pS to 1 nS in symmetric 150 mM KCl depending on experimental conditions. Single channels were inhibited by lanthanum with an unusually high Hill coefficient (8.4 ± 1.2). Transition to low-conductance states (<250 pS) was favored by increased membrane polarization. High-conductance states (>250 pS) may reflect conformations important for genetic transformability, or "competence," of the bacterial cells, which requires the presence of the PHB/Ca^{2+}/polyP complex in the membrane.

More importantly, a similar polyP/Ca^{2+}/PHB channel was isolated from rat liver mitochondria (Pavlov et al. 2005b). In addition to the cation-selective conductance state, this mitochondrial complex also demonstrated a high-conductance, weakly selective, voltage-dependent state. These properties in many ways reflected the behavior of the mPTP as seen in patch-clamp studies of native mitochondrial membranes (Szabo and Zoratti 1991; Kinnally et al. 1991). Interestingly, the polyP/Ca^{2+}/PHB channel of bacterial origin also has this high-conductance state (Pavlov et al.

2005a). The transition of the channel into a high-conductance state would most likely be deleterious for bacterial organisms, raising the question whether most of the time the bacterial channel is either closed or is in the low-conductance cationic state. The different bacterial conductance states are reminiscent of conductance states proposed for the mPTP (Ichas et al. 1997; Ichas and Mazat 1998; Huser and Blatter 1999; Huser et al. 1998). The parallels between bacteria and mitochondria also suggest that similar cationic channels may play a role in normal mitochondrial function. In support of such notion, the polyP/Ca^{2+}/PHB complex has been detected in various eukaryotic organisms and cellular compartments, suggesting a potential physiological role (Reusch 1989). Currently, the direct test whether a polyP/Ca^{2+}/PHB complex indeed forms the pore part of the mPTP in intact mitochondria remains an experimental challenge. Nonetheless, the idea that the presence of polyP in intact mitochondria is an essential condition for mPTP opening remains an intriguing hypothesis. Indeed, it was shown that mitochondria of cultured cells with reduced levels of polyP are more resistant toward Ca^{2+}-induced mPTP opening (Abramov et al. 2007). We demonstrated that enzymatic depletion of polyP from cells achieved by overexpression of the mitochondrial-targeting sequence GFP yeast exopolyphosphatase (MTS-GFP-scPPX1) (Fig. 7.2a, b) resulted in decreased openings of Ca^{2+}-induced mPTP in permeabilized rabbit ventricular cardiomyocytes. In contrast to non-excitable cells (Abramov et al. 2007), polyP depletion did not affect the ability of mitochondria to accumulate Ca^{2+} (Fig. 7.2c, d), however, significantly decreased Ca^{2+}-induced openings of mPTP (Fig. 7.3a, b) and prevented Ca^{2+}-induced loss of $\Delta\Psi_m$ (Fig. 7.3c–e) indicating that polyP is a potent activator of Ca^{2+}-induced mPTP. The study in non-excitable cells did not measure mPTP activity directly; however, the authors also found that the high Ca^{2+} induced a CsA-sensitive drop in $\Delta\Psi_m$ in control but not in polyP-depleted cells. Altogether, these data demonstrate that inorganic polyP is a potent activator of Ca^{2+}-induced mPTP in both excitable and non-excitable cells.

On the other hand, polyP depletion did not protect from mPTP opening induced by cell exposure to high laser power (i.e., presumably leading to the increased ROS generation) in TMRM-loaded cells (Abramov et al. 2007). The data from our group revealed (Seidlmayer et al. 2015) that when mPTP activity was monitored in conditions of simulated ischemia-reperfusion accompanied by massive ROS generation, polyP depletion was not able to prevent mPTP opening and cell death. In fact, as we demonstrated earlier (Seidlmayer et al. 2015), ROS generation and cell death were significantly increased under conditions of ischemia-reperfusion in polyP-depleted cells. We detected different modes in mPTP activity during ischemia and reperfusion, and these modes were affected differently by polyP. In agreement with our data obtained on permeabilized cells (Seidlmayer et al. 2012b), polyP depletion prevented Ca^{2+}-induced low-conductance mPTP mode observed during ischemia; however, it did not affect ROS-mediated mPTP opening in the high-conductance mode during reperfusion (Fig. 7.4a, b). It is important to emphasize that polyP-mediated mPTP opening during ischemia was not associated with cell death (Fig. 7.4c), while ROS-mediated mPTP opening during reperfusion led to the increase in cell death. Furthermore, cell death during reperfusion was significantly

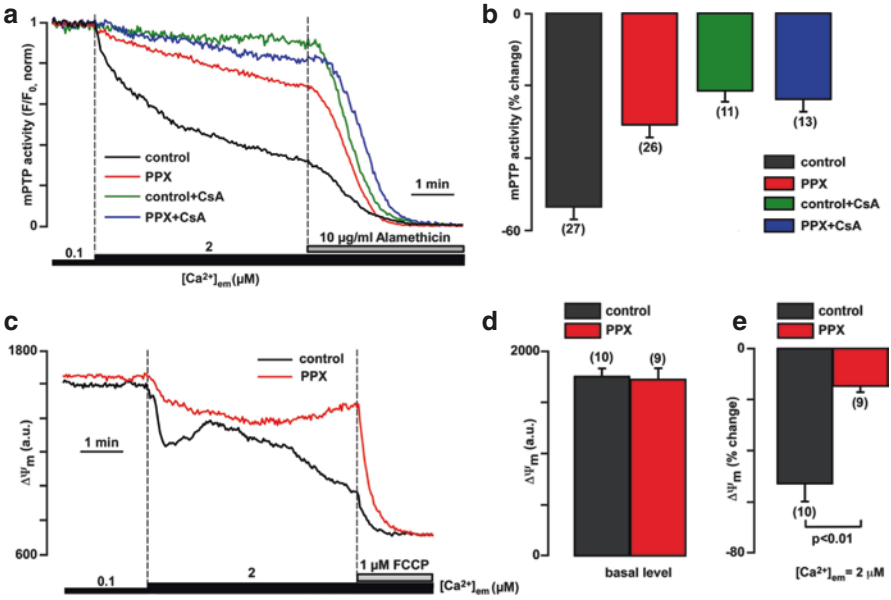

Fig. 7.3 PolyP depletion prevents opening of the permeability transition pore induced by mitochondrial Ca^{2+} overload. (**a**) Original recordings of mPTP opening using calcein red release from mitochondria of permeabilized control (*black*), polyP-depleted (PPX-expressing, *red*), and cyclosporine A (CsA)-treated control (*blue*) and polyP-depleted (*green*) myocytes. After permeabilization cells were exposed to 2 µM Ca^{2+} and 10 µg/ml, alamethicin was added at the end of the experiment to achieve the maximal calcein *red* release from mitochondria. (**b**) Summary of calcein *red* release (as percent of total release obtained after alamethicin addition) from mitochondria measured at the end of 2 µM Ca^{2+} exposure in control, PPX-expressing, CsA-treated control cells and CsA-treated PPX-expressing cells. (**c**) Original recordings of TMRM fluorescence in permeabilized rabbit ventricular myocytes upon elevation of $[Ca^{2+}]_{em}$ from 0.1 to 2 µM and subsequent addition of 1 µM FCCP in control (*black*) and polyP-depleted (PPX-expressing, *red*) myocytes. (**d**) Average values reflecting the basal levels of TMRM fluorescence in control and polyP-depleted cells. (**e**) Average TMRM fluorescence decrease, measured at the end of exposure to 2 µM Ca^{2+} as % decrease from initial levels in control and polyP-depleted cells (Modified with permission from Seidlmayer et al. 2012b)

enhanced in polyP-depleted cells (Fig. 7.4c). These exciting findings indicate that polyP has a dual effect on mPTP activity – promoting the transient opening of Ca^{2+}-induced mPTP opening which can prevent mitochondria from Ca^{2+} overload. On the other hand, polyP was required for protection against oxidative stress-induced mPTP opening and cell death. It is unclear at this point, whether this effect of polyP was related to the recently discovered chaperone activity of polyP or the direct effect of polyP on mPTP. Intriguingly, it was reported that dimers of the F_1F_0-ATP synthase can form channels with characteristics similar to the mPTP (Giorgio et al. 2013); however, the molecular details of channel formation by F_1F_0-ATP synthase remain unclear. It was suggested that subunit c of the F_1F_0-ATP synthase could be a key component of the mPTP (Bonora et al. 2013; Azarashvili et al. 2002; Alavian et al. 2014). Interestingly, an interaction of polyP/Ca^{2+}/PHB complex with subunit c

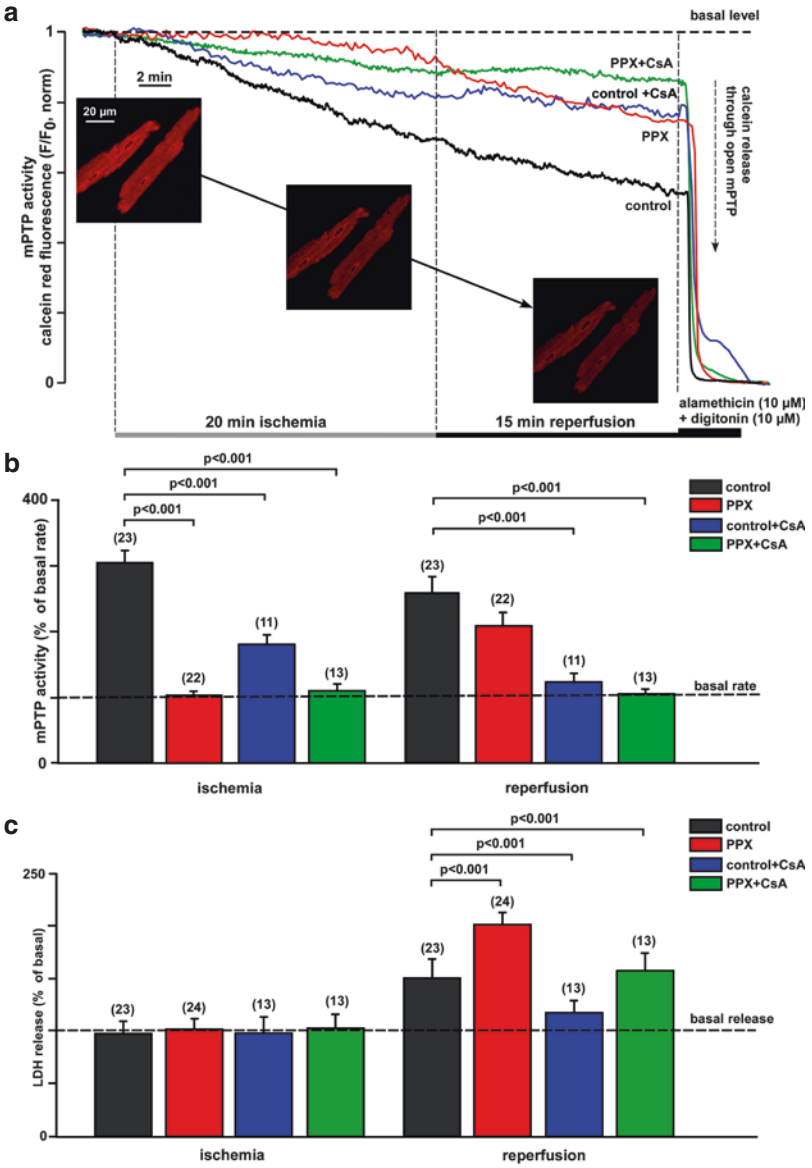

Fig. 7.4 Effects of polyP depletion and cyclosporin A (CsA) on mPTP activity and necrotic cell death following ischemia-reperfusion. (**a**) Normalized traces of calcein *red* fluorescence changes during 20 min ischemia followed by 15 min of reperfusion in control (*black*) and polyP-depleted (*red*) cells in the absence or presence of 1 µM of CsA (mPTP inhibitor). *Insets* show images of calcein-loaded cardiomyocytes before and after exposure to ischemia-reperfusion. (**b**) Summary of calcein *red* release from mitochondria (as percent of basal rate) at the end of ischemia and reperfusion. (**c**) Cell death (% of lactate dehydrogenase (LDH) release with respect to basal LDH release rates) measured at the end of ischemia and reperfusion in control and polyP-depleted (PPX) cells in the absence or presence of 1 µM CsA. *n* is the number of hearts used in each experiment (Modified with permission from Seidlmayer et al. 2015)

of F_1F_0-ATP synthase was reported back in 2005 (Pavlov et al. 2005b), and therefore it is possible that polyP could provide a fine tuning of mPTP regulation or actually mediate Ca^{2+} transfer through mPTP. The physiological importance of transient (low-conductance) mPTP opening, which does not lead to cell death (Zoratti and Szabo 1995), has been suggested to mediate ischemic preconditioning-induced protection (Hausenloy et al. 2004, 2009) via (i) regulation of the mitochondrial matrix Ca^{2+} concentration (Ichas et al. 1997), (ii) induction of mild mitochondrial uncoupling (Zago et al. 2000), and (iii) regulation of mitochondrial ROS release (Zorov et al. 2000). We suggest that inorganic polyP can also contribute to the mPTP opening in a low-conductance mode. Furthermore, our findings show that depletion of polyP was associated with enhanced cell death on reperfusion, indicating that stimulation of polyP production rather than inhibition of Ca^{2+} uptake on reperfusion could be beneficial for cardioprotection.

7.8 Concluding Remarks

The current data reviewed here are consistent with a key role of polyP in activation of the mitochondrial permeability transition pore and in regulation of stress-induced cell death in both excitable and non-excitable cells. Based on the presented evidence, we suggest that at least two pools of polyP exist in mitochondria; one pool is incorporated directly in the inner mitochondrial membrane as a polyP-PHB-Ca^{2+} complex, and the other pool is present in the mitochondrial matrix and is not bound to PHB. This free pool of polyP most probably is responsible for the metabolic effects of polyP in mitochondria and Ca^{2+} buffering. We speculate that endogenous levels of mitochondrial polyP reflect the ability of the cell to survive stress conditions. Mitochondrial polyP concentration is subject to remodeling processes in heart failure and during ischemia-reperfusion. Definitely, more studies are required to elucidate the exact mechanisms of polyP actions in mitochondria and its protein targets.

Funding This work was supported by the American Heart Association Grant-in-Aid (15GRNT25090220) to Elena N. Dedkova.

References

Abramov AY, Fraley C, Diao CT, Winkfein R, Colicos MA, Duchen MR, French RJ, Pavlov E (2007) Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death. Proc Natl Acad Sci U S A 104(46):18091–18096. doi:0708959104 [pii] 10.1073/pnas.0708959104

Ahn K, Kornberg A (1990) Polyphosphate kinase from Escherichia coli. Purification and demonstration of a phosphoenzyme intermediate. J Biol Chem 265(20):11734–11739

Akiyama M, Crooke E, Kornberg A (1992) The polyphosphate kinase gene of Escherichia coli. Isolation and sequence of the ppk gene and membrane location of the protein. J Biol Chem 267(31):22556–22561

Akiyama M, Crooke E, Kornberg A (1993) An exopolyphosphatase of Escherichia coli. The enzyme and its ppx gene in a polyphosphate operon. J Biol Chem 268(1):633–639

Alavian KN, Beutner G, Lazrove E, Sacchetti S, Park HA, Licznerski P, Li H, Nabili P, Hockensmith K, Graham M, Porter GA Jr, Jonas EA (2014) An uncoupling channel within the c-subunit ring of the F1FO ATP synthase is the mitochondrial permeability transition pore. Proc Natl Acad Sci U S A 111(29):10580–10585. doi:10.1073/pnas.1401591111

Andreeva NA, Okorokov LA (1993) Purification and characterization of highly active and stable polyphosphatase from Saccharomyces cerevisiae cell envelope. Yeast 9(2):127–139. doi:10.1002/yea.320090204

Aschar-Sobbi R, Abramov AY, Diao C, Kargacin ME, Kargacin GJ, French RJ, Pavlov E (2008) High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. J Fluoresc 18(5):859–866. doi:10.1007/s10895-008-0315-4

Ault-Riche D, Fraley CD, Tzeng CM, Kornberg A (1998) Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in Escherichia coli. J Bacteriol 180(7):1841–1847

Azarashvili TS, Tyynela J, Odinokova IV, Grigorjev PA, Baumann M, Evtodienko YV, Saris NE (2002) Phosphorylation of a peptide related to subunit c of the F0F1-ATPase/ATP synthase and relationship to permeability transition pore opening in mitochondria. J Bioenerg Biomembr 34(4):279–284

Belozerskii AN, Kulaev IS (1957) Polyphosphates and their significance in the development of Aspergillus niger. Biokhimiia 22(1–2):29–39

Bonora M, Bononi A, De Marchi E, Giorgi C, Lebiedzinska M, Marchi S, Patergnani S, Rimessi A, Suski JM, Wojtala A, Wieckowski MR, Kroemer G, Galluzzi L, Pinton P (2013) Role of the c subunit of the FO ATP synthase in mitochondrial permeability transition. Cell Cycle 12(4):674–683. doi:10.4161/cc.23599

Brown MR, Kornberg A (2004) Inorganic polyphosphate in the origin and survival of species. Proc Natl Acad Sci U S A 101(46):16085–16087. doi:10.1073/pnas.0406909101

Castuma CE, Huang R, Kornberg A, Reusch RN (1995) Inorganic polyphosphates in the acquisition of competence in Escherichia coli. J Biol Chem 270(22):12980–12983

Chakrabarty AM (1998) Nucleoside diphosphate kinase: role in bacterial growth, virulence, cell signalling and polysaccharide synthesis. Mol Microbiol 28(5):875–882

Cowling RT, Birnboim HC (1994) Incorporation of [32P]orthophosphate into inorganic polyphosphates by human granulocytes and other human cell types. J Biol Chem 269(13):9480–9485

Crooke E, Akiyama M, Rao NN, Kornberg A (1994) Genetically altered levels of inorganic polyphosphate in Escherichia coli. J Biol Chem 269(9):6290–6295

Das S, Lengweiler UD, Seebach D, Reusch RN (1997) Proof for a nonproteinaceous calcium-selective channel in Escherichia coli by total synthesis from (R)-3-hydroxybutanoic acid and inorganic polyphosphate. Proc Natl Acad Sci U S A 94(17):9075–9079

Dedkova EN (2016) Inorganic polyphosphate in cardiac myocytes: from bioenergetics to the permeability transition pore and cell survival. Biochem Soc Trans 44(1):25–34. doi:10.1042/BST20150218

Dedkova EN, Blatter LA (2012) Measuring mitochondrial function in intact cardiac myocytes. J Mol Cell Cardiol 52(1):48–61. doi:S0022-2828(11)00412-3 [pii] 10.1016/j.yjmcc.2011.08.030

Dedkova EN, Seidlmayer LK, Blatter LA (2013) Mitochondria-mediated cardioprotection by trimetazidine in rabbit heart failure. J Mol Cell Cardiol 59:41–54. doi:10.1016/j.yjmcc.2013.01.016

Dedkova EN, Blatter LA (2014) Role of beta-hydroxybutyrate, its polymer poly-beta-hydroxybutyrate and inorganic polyphosphate in mammalian health and disease. Front Physiol 5:260. doi:10.3389/fphys.2014.00260

Demirkhanyan L, Palmer IP, Boyd W, Sondergaard CS, Grimsrud K, Griffiths LG, Bossuyt J, Bers DM, Zakharian E, Dedkova E (2016) Formation of polyphosphate/poly-beta-hydroxybutyrate granular-like complexes in heart failure myocytes. Biophys J 2016(110):3

Docampo R, de Souza W, Miranda K, Rohloff P, Moreno SN (2005) Acidocalcisomes – conserved from bacteria to man. Nat Rev Microbiol 3(3):251–261. doi:nrmicro1097 [pii] 10.1038/nrmicro1097

Duan RD (2006) Alkaline sphingomyelinase: an old enzyme with novel implications. Biochim Biophys Acta 1761(3):281–291. doi:10.1016/j.bbalip.2006.03.007

EEFS (2013) (European Food Safety Authority), 2013. Assessment of one published review on health risks associated with phosphate additives in food EFSA Journal 11(3444):1–27

Gan XT, Ettinger G, Huang CX, Burton JP, Haist JV, Rajapurohitam V, Sidaway JE, Martin G, Gloor GB, Swann JR, Reid G, Karmazyn M (2014) Probiotic administration attenuates myocardial hypertrophy and heart failure after myocardial infarction in the rat. Circ Heart Fail 7(3):491–499. doi:10.1161/CIRCHEARTFAILURE.113.000978

Giorgio V, von Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, Glick GD, Petronilli V, Zoratti M, Szabo I, Lippe G, Bernardi P (2013) Dimers of mitochondrial ATP synthase form the permeability transition pore. Proc Natl Acad Sci U S A 110(15):5887–5892. doi:10.1073/pnas.1217823110

Gomez-Garcia MR, Kornberg A (2004) Formation of an actin-like filament concurrent with the enzymatic synthesis of inorganic polyphosphate. Proc Natl Acad Sci U S A 101(45):15876–15880. doi:10.1073/pnas.0406923101

Graham T (1833) Researches on the arsenates, phosphates and modifications of phosphoric acid. Philos Trans Roy Soc 123:253–356

Gray MJ, Wholey WY, Wagner NO, Cremers CM, Mueller-Schickert A, Hock NT, Krieger AG, Smith EM, Bender RA, Bardwell JC, Jakob U (2014) Polyphosphate is a primordial chaperone. Mol Cell 53(5):689–699. doi:10.1016/j.molcel.2014.01.012

Hausenloy D, Wynne A, Duchen M, Yellon D (2004) Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. Circulation 109(14):1714–1717. doi:10.1161/01.CIR.0000126294.81407.7D

Hausenloy DJ, Ong SB, Yellon DM (2009) The mitochondrial permeability transition pore as a target for preconditioning and postconditioning. Basic Res Cardiol 104(2):189–202. doi:10.1007/s00395-009-0010-x

Hooley P, Whitehead MP, Brown MR (2008) Eukaryote polyphosphate kinases: is the 'Kornberg' complex ubiquitous? Trends Biochem Sci 33(12):577–582. doi:10.1016/j.tibs.2008.09.007

Huang R, Reusch RN (1995) Genetic competence in Escherichia coli requires poly-beta-hydroxybutyrate/calcium polyphosphate membrane complexes and certain divalent cations. J Bacteriol 177(2):486–490

Huser J, Blatter LA (1999) Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. Biochem J 343(Pt 2):311–317

Huser J, Rechenmacher CE, Blatter LA (1998) Imaging the permeability pore transition in single mitochondria. Biophys J 74(4):2129–2137

Ichas F, Mazat JP (1998) From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim Biophys Acta 1366(1–2):33–50

Ichas F, Jouaville LS, Mazat JP (1997) Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. Cell 89(7):1145–1153

Ishige K, Zhang H, Kornberg A (2002) Polyphosphate kinase (PPK2), a potent, polyphosphate-driven generator of GTP. Proc Natl Acad Sci U S A 99(26):16684–16688. doi:10.1073/pnas.262655299

Jin H, Xu CX, Lim HT, Park SJ, Shin JY, Chung YS, Park SC, Chang SH, Youn HJ, Lee KH, Lee YS, Ha YC, Chae CH, Beck GR Jr, Cho MH (2009) High dietary inorganic phosphate increases lung tumorigenesis and alters Akt signaling. Am J Respir Crit Care Med 179(1):59–68. doi:10.1164/rccm.200802-306OC

Kampinga HH (2014) Chaperoned by prebiotic inorganic polyphosphate molecules: an ancient transcription-independent mechanism to restore protein homeostasis. Mol Cell 53(5):685–687. doi:10.1016/j.molcel.2014.02.023

Kim HY, Schlictman D, Shankar S, Xie Z, Chakrabarty AM, Kornberg A (1998) Alginate, inorganic polyphosphate, GTP and ppGpp synthesis co-regulated in Pseudomonas aeruginosa: implications for stationary phase survival and synthesis of RNA/DNA precursors. Mol Microbiol 27(4):717–725

Kim KS, Rao NN, Fraley CD, Kornberg A (2002) Inorganic polyphosphate is essential for long-term survival and virulence factors in Shigella and Salmonella spp. Proc Natl Acad Sci U S A 99(11):7675–7680. doi:10.1073/pnas.112210499

Kinnally KW, Campo ML, Tedeschi H (1989) Mitochondrial channel activity studied by patch-clamping mitoplasts. J Bioenerg Biomembr 21(4):497–506

Kinnally KW, Zorov D, Antonenko Y, Perini S (1991) Calcium modulation of mitochondrial inner membrane channel activity. Biochem Biophys Res Commun 176(3):1183–1188

Kornberg SR (1957) Adenosine triphosphate synthesis from polyphosphate by an enzyme from Escherichia coli. Biochim Biophys Acta 26(2):294–300

Kornberg A (1995) Inorganic polyphosphate: toward making a forgotten polymer unforgettable. J Bacteriol 177(3):491–496

Kornberg A (1999) Inorganic polyphosphate: a molecule of many functions. Prog Mol Subcell Biol 23:1–18

Kornberg A, Rao NN, Ault-Riche D (1999) Inorganic polyphosphate: a molecule of many functions. Annu Rev Biochem 68:89–125. doi:10.1146/annurev.biochem.68.1.89

Kulaev IS, Belozerskii AN (1957) P32 in the study of physiological role of polyphosphates in the process of development of Aspergillus niger. Biokhimiia 22(3):587–596

Kulaev IS, Bobyk MA (1971) Detection in Neurospora crassa of a new enzyme – 1,3-diphosphoglycerate: polyphosphate phosphotransferase. Biokhimiia 36(2):426–429

Kulaev IS, Vagabov VM (1983) Polyphosphate metabolism in micro-organisms. Adv Microb Physiol 24:83–171

Kulaev IS, Kritskii MS, Belozerskii AN (1960) Metabolism of polyphosphates and of certain other phosphorus compounds during the process of development of fruit bodies in the mushroom Agaricus bisporus L. Biokhimiia 25:735–748

Kulaev I, Vagabov V, Kulakovskaya T (1999) New aspects of inorganic polyphosphate metabolism and function. J Biosci Bioeng 88(2):111–129. doi:S1389-1723(99)80189-3 [pii]

Kulakovskaya T, Kulaev I (2013) Enzymes of inorganic polyphosphate metabolism. Prog Mol Subcell Biol 54:39–63. doi:10.1007/978-3-642-41004-8_3

Kulakovskaya TV, Vagabov VM, Kulaev IS (2012) Inorganic polyphosphate in industry, agriculture and medicine: modern state and outlook. Process Biochem 47:1–10

Kumble KD, Kornberg A (1995) Inorganic polyphosphate in mammalian cells and tissues. J Biol Chem 270(11):5818–5822

Kumble KD, Kornberg A (1996) Endopolyphosphatases for long chain inorganic polyphosphate in yeast and mammals. J Biol Chem 271(43):27146–27151

Kumble KD, Ahn K, Kornberg A (1996) Phosphohistidyl active sites in polyphosphate kinase of Escherichia coli. Proc Natl Acad Sci U S A 93(25):14391–14395

Lam V, Su J, Koprowski S, Hsu A, Tweddell JS, Rafiee P, Gross GJ, Salzman NH, Baker JE (2012) Intestinal microbiota determine severity of myocardial infarction in rats. FASEB J Off Publ Fed Am Soc Exp Biol 26(4):1727–1735. doi:10.1096/fj.11-197921

Lorenz B, Schroder HC (2001) Mammalian intestinal alkaline phosphatase acts as highly active exopolyphosphatase. Biochim Biophys Acta 1547(2):254–261

Lorenz B, Muller WE, Kulaev IS, Schroder HC (1994) Purification and characterization of an exopolyphosphatase from Saccharomyces cerevisiae. J Biol Chem 269(35):22198–22204

Lorenz B, Munkner J, Oliveira MP, Kuusksalu A, Leitao JM, Muller WE, Schroder HC (1997) Changes in metabolism of inorganic polyphosphate in rat tissues and human cells during development and apoptosis. Biochim Biophys Acta 1335(1–2):51–60

Lynn WS, Brown RH (1963) Synthesis of polyphosphate by rat liver mitochondria. Biochem Biophys Res Commun 11:367–371

Maraeva OB, Kolot MN, Nesmeianova MA, Kulaev IS (1979) Interrelationship between metabolic and genetic regulation of alkaline phosphatase and poly- and pyrophosphatases. Biokhimiia 44(4):715–719

Matsuzaki H, Masuyama R, Uehara M, Nakamura K, Suzuki K (2001) Greater effect of dietary potassium tripolyphosphate than of potassium dihydrogenphosphate on the nephrocalcinosis

and proximal tubular function in female rats from the intake of a high-phosphorus diet. Biosci Biotechnol Biochem 65(4):928–934. doi:10.1271/bbb.65.928

McCafferty K, Byrne C, Yaqoob M (2012) Intestinal microbiota determine severity of myocardial infarction in rats. FASEB J Off Publ Fed Am Soc Exp Biol 26(11):4388. doi:10.1096/fj.12-1102LTR, author reply 4388–4389

Nesmeianova MA, Dmitriev AD, Kulaev IS (1973) High-molecular polyphosphates and enzymes of polyphosphate metabolism in the process of growth of an Escherichia coli culture. Mikrobiologiia 42(2):213–219

Nesmeianova MA, Dmitriev AD, Kulaev IS (1974) Exogenous orthophosphate regulation of phosphorus metabolism enzymes and polyphosphate levels in Escherichia coli K-12. Mikrobiologiia 43(2):227–234

Ohashi K, Kawai S, Murata K (2012) Identification and characterization of a human mitochondrial NAD kinase. Nat Commun 3:1248. doi:10.1038/ncomms2262

Pavlov E, Grimbly C, Diao CT, French RJ (2005a) A high-conductance mode of a poly-3-hydroxybutyrate/calcium/polyphosphate channel isolated from competent Escherichia coli cells. FEBS Lett 579(23):5187–5192. doi:S0014-5793(05)01027-6 [pii] 10.1016/j.febslet.2005.08.032

Pavlov E, Zakharian E, Bladen C, Diao CT, Grimbly C, Reusch RN, French RJ (2005b) A large, voltage-dependent channel, isolated from mitochondria by water-free chloroform extraction. Biophys J 88(4):2614–2625. doi:S0006-3495(05)73316-6 [pii] 10.1529/biophysj.104.057281

Pavlov E, Aschar-Sobbi R, Campanella M, Turner RJ, Gomez-Garcia MR, Abramov AY (2010) Inorganic polyphosphate and energy metabolism in mammalian cells. J Biol Chem 285(13):9420–9428. doi:M109.013011 [pii] 10.1074/jbc.M109.013011

Pisoni RL, Lindley ER (1992) Incorporation of [32P]orthophosphate into long chains of inorganic polyphosphate within lysosomes of human fibroblasts. J Biol Chem 267(6):3626–3631

Pollak N, Niere M, Ziegler M (2007) NAD kinase levels control the NADPH concentration in human cells. J Biol Chem 282(46):33562–33571. doi:10.1074/jbc.M704442200

Rao NN, Gomez-Garcia MR, Kornberg A (2009) Inorganic polyphosphate: essential for growth and survival. Annu Rev Biochem 78:605–647. doi:10.1146/annurev.biochem.77.083007.093039

Rashid MH, Kornberg A (2000) Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 97(9):4885–4890. doi:10.1073/pnas.060030097

Rashid MH, Rao NN, Kornberg A (2000a) Inorganic polyphosphate is required for motility of bacterial pathogens. J Bacteriol 182(1):225–227

Rashid MH, Rumbaugh K, Passador L, Davies DG, Hamood AN, Iglewski BH, Kornberg A (2000b) Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 97(17):9636–9641. doi:10.1073/pnas.170283397

Reusch RN (1989) Poly-beta-hydroxybutyrate/calcium polyphosphate complexes in eukaryotic membranes. Proc Soc Exp Biol Med 191(4):377–381

Reusch RN (2000) Transmembrane ion transport by polyphosphate/poly-(R)-3-hydroxybutyrate complexes. Biochemistry (Mosc) 65(3):280–295

Reusch RN, Sadoff HL (1988) Putative structure and functions of a poly-beta-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc Natl Acad Sci U S A 85(12):4176–4180

Reusch RN, Huang R, Kosk-Kosicka D (1997) Novel components and enzymatic activities of the human erythrocyte plasma membrane calcium pump. FEBS Lett 412(3):592–596

Ruiz FA, Lea CR, Oldfield E, Docampo R (2004) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 279(43):44250–44257. doi:10.1074/jbc.M406261200

Sedova M, Dedkova EN, Blatter LA (2006) Integration of rapid cytosolic Ca^{2+} signals by mitochondria in cat ventricular myocytes. Am J Physiol Cell Physiol 291(5):C840–C850

Segawa S, Fujiya M, Konishi H, Ueno N, Kobayashi N, Shigyo T, et al (2011) Probiotic-derived polyphosphate enhances the epithelial barrier function and maintains intestinal homeostasis through integrin-p38 MAPK pathway. PLoS ONE 6:e23278. doi:10.1371/journal.pone.0023278

Seidlmayer LK, Blatter LA, Pavlov E, Dedkova EN (2012a) Inorganic polyphosphate – an unusual suspect of the mitochondrial permeability transition mystery. Channels (Austin) 6(6):463–467. doi:10.4161/chan.21939

Seidlmayer LK, Gomez-Garcia MR, Blatter LA, Pavlov E, Dedkova EN (2012b) Inorganic polyphosphate is a potent activator of the mitochondrial permeability transition pore in cardiac myocytes. J Gen Physiol 139(5):321–331. doi:jgp.201210788 [pii] 10.1085/jgp.201210788

Seidlmayer LK, Juettner VV, Kettlewell S, Pavlov EV, Blatter LA, Dedkova EN (2015) Distinct mPTP activation mechanisms in ischaemia-reperfusion: contributions of Ca^{2+}, ROS, pH, and inorganic polyphosphate. Cardiovasc Res 106(2):237–248. doi:10.1093/cvr/cvv097

Sen AR, Naveena BM, Muthukumar M, Babji Y, Murthy TR (2005) Effect of chilling, polyphosphate and bicarbonate on quality characteristics of broiler breast meat. Br Poult Sci 46(4):451–456. doi:10.1080/00071660500191072

Solesio ME, Demirkhanyan L, Zakharian E, Pavlov EV (2016) Contribution of inorganic polyphosphate towards regulation of mitochondrial free calcium. Biochim Biophys Acta 1860(6):1317–1325. doi:10.1016/j.bbagen.2016.03.020

Sureka K, Sanyal S, Basu J, Kundu M (2009) Polyphosphate kinase 2: a modulator of nucleoside diphosphate kinase activity in mycobacteria. Mol Microbiol 74(5):1187–1197. doi:10.1111/j.1365-2958.2009.06925.x

Szabo I, Zoratti M (1991) The giant channel of the inner mitochondrial membrane is inhibited by cyclosporin A. J Biol Chem 266(6):3376–3379

Takeda E, Yamamoto H, Yamanaka-Okumura H, Taketani Y (2014) Increasing dietary phosphorus intake from food additives: potential for negative impact on bone health. Adv Nutr 5(1):92–97. doi:10.3945/an.113.004002

Tammenkoski M, Koivula K, Cusanelli E, Zollo M, Steegborn C, Baykov AA, Lahti R (2008) Human metastasis regulator protein H-prune is a short-chain exopolyphosphatase. Biochemistry 47(36):9707–9713. doi:10.1021/bi8010847

Vasant G, Krishnamurthy VN, Sudha G, Manik D, Kalyani P, Borlaug N (2009) The fertilizer encyclopedia. John Wiley & Sons, Inc., Hoboken, New Jersey, pp 1–872

Watari E, Taketani Y, Kitamura T, Tanaka T, Ohminami H, Abuduli M, Harada N, Yamanaka-Okumura H, Yamamoto H, Takeda E (2015) Fluctuating plasma phosphorus level by changes in dietary phosphorus intake induces endothelial dysfunction. J Clin Biochem Nutr 56(1):35–42. doi:10.3164/jcbn.14-96

Wei AC, Liu T, Winslow RL, O'Rourke B (2012) Dynamics of matrix-free Ca^{2+} in cardiac mitochondria: two components of Ca^{2+} uptake and role of phosphate buffering. J Gen Physiol 139(6):465–478. doi:jgp.201210784 [pii] 10.1085/jgp.201210784

Whitehead MP, Hooley P, W Brown MR (2013) Horizontal transfer of bacterial polyphosphate kinases to eukaryotes: implications for the ice age and land colonisation. BMC Res Notes 6:221. doi:10.1186/1756-0500-6-221

Whitehead MP, Eagles L, Hooley P, Brown MR (2014) Most bacteria synthesize polyphosphate by unknown mechanisms. Microbiology 160(Pt 5):829–831. doi:10.1099/mic.0.075366-0

Wood HG, Clark JE (1988) Biological aspects of inorganic polyphosphates. Annu Rev Biochem 57:235–260. doi:10.1146/annurev.bi.57.070188.001315

Wurst H, Kornberg A (1994) A soluble exopolyphosphatase of Saccharomyces cerevisiae. Purification and characterization. J Biol Chem 269(15):10996–11001

Wurst H, Shiba T, Kornberg A (1995) The gene for a major exopolyphosphatase of Saccharomyces cerevisiae. J Bacteriol 177(4):898–906

Zago EB, Castilho RF, Vercesi AE (2000) The redox state of endogenous pyridine nucleotides can determine both the degree of mitochondrial oxidative stress and the solute selectivity of the permeability transition pore. FEBS Lett 478(1–2):29–33

Zhang H, Ishige K, Kornberg A (2002) A polyphosphate kinase (PPK2) widely conserved in bacteria. Proc Natl Acad Sci U S A 99(26):16678–16683. doi:10.1073/pnas.262655199

Zhang H, Gomez-Garcia MR, Brown MR, Kornberg A (2005) Inorganic polyphosphate in Dictyostelium discoideum: influence on development, sporulation, and predation. Proc Natl Acad Sci U S A 102(8):2731–2735. doi:10.1073/pnas.0500023102

Zhang H, Gomez-Garcia MR, Shi X, Rao NN, Kornberg A (2007) Polyphosphate kinase 1, a conserved bacterial enzyme, in a eukaryote, Dictyostelium discoideum, with a role in cytokinesis. Proc Natl Acad Sci U S A 104(42):16486–16491. doi:10.1073/pnas.0706847104

Zhu Y, Huang W, Lee SS, Xu W (2005) Crystal structure of a polyphosphate kinase and its implications for polyphosphate synthesis. EMBO Rep 6:681–687

Zoratti M, Szabo I (1995) The mitochondrial permeability transition. Biochim Biophys Acta 1241(2):139–176. doi:0304-4157(95)00003-A [pii]

Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ (2000) Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med 192(7):1001–1014

Role of Inorganic Polyphosphate in the Cells of the Mammalian Brain

8

Artyom Y. Baev, Plamena R. Angelova, and Andrey Y. Abramov

Abstract

Inorganic polyphosphate (polyP) is a polymer molecule of variable chain length, formed of linearly arranged orthophosphate residues that are linked through high-energy phosphoanhydride bonds identical to the ones found in ATP. PolyP has also been found in mammalian cells – it is important for blood coagulation, osteoclast function, immune response, etc. In the brain polyP is essential for precise functioning of mitochondria – from significance for the energy metabolism and mitochondrial calcium handling to the activation of permeability transition pore (PTP). PolyP has been shown to be a gliotransmitter, enabling signaling between astrocytes via activation of P2Y1 receptors. Thus, polyP plays many important roles in mammalian brain cells, ranging from signal transduction, mitochondrial metabolism, and activation of ion channels to involvement in PTP opening and triggering of cell death.

Inorganic polyphosphate (polyP) is an ancient polymer molecule with various chain lengths of orthophosphates, which is present and plays multiple roles in all kinds of organisms. The German scientist Leo Liebermann was the first to find and isolate inorganic polyphosphates from yeast (Liebermann 1888). However, the real interest to this molecule grew only in the second part of the twentieth century, after the development of certain methods of polyP detection. First studies have been made in

A.Y. Baev
Biology Faculty, National University of Uzbekistan, Tashkent, Uzbekistan

P.R. Angelova • A.Y. Abramov (✉)
Department of Molecular Neuroscience, UCL Institute of Neurology, Queen Square, WC1N 3BG London, UK
e-mail: a.abramov@ucl.ac.uk

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_8

microorganisms and unicellular eukaryotes, which contain high concentration of this polymer. For many years, it has been thought that polyP has no specific function in higher organisms, as far as no enzymatic systems responsible for synthesis and degradation of this molecule have been found. However, in the last decade a growing body of evidence in the literature has pointed out to many faces of activity of polyP in higher organisms. Since then it has been found that polyphosphate is involved in many cellular functions in the mammalian organisms like angiogenesis (Han et al. 2007), apoptosis (Hernandez-Ruiz et al. 2006), osteoblast function (Kawazoe et al. 2004), blood clotting and inflammation (Morrissey 2012), cell bioenergetics (Pavlov et al. 2010), ion channel function (Kim and Cavanaugh 2007; Zakharian et al. 2009), PTP formation (Abramov et al. 2007; Seidlmayer et al. 2012), nuclear transcription (Jimenez-Nunez et al. 2012), cell signaling (Holmstrom et al. 2013), etc.

The concentration of polyP in the brain varies from 25 to 120 μM depending on the cell type and on the physiological conditions (Kumble and Kornberg 1995; Gabel and Thomas 1971). Importantly, the concentration of the polyP in mammalian brain is increasing within the time of development (Lorenz et al. 1997).

Inorganic polyphosphates are big linear anions consisting of orthophosphate residues, which are linked together via high-energy phosphoanhydride bonds like in the ATP molecule. Because of the structural similarity between ATP and polyP, there was always a question about the possibility for polyP to implement similar functions as ATP in eukaryotic cells. There has been a mystery around the localization of polyP synthesis and about the identity of the enzymatic pathways involved in this process in higher organisms. In 2010, we demonstrated that the level of polyP dynamically changes dependent on mitochondrial respiration and phosphorylation. The level of polyP decreased during inhibition of respiratory chain activity by different inhibitors. The same effect has been observed in the presence of an uncoupler of the mitochondrial respiration FCCP (Pavlov et al. 2010; Angelova et al. 2014). At the same time addition of glutamate, Me-succinate, and pyruvate (substrates for respiratory chain, all at 5 mM) to the system increased concentration of polyP dramatically. It is interesting that the increase in polyP level appeared within the next 30–40 s following addition of substrates (Pavlov et al. 2010). Interestingly, the inhibitor of F_1F_0 ATP synthase oligomycin had opposite effect on different cell types. In primary astrocytes the level of polyP decreased with ~55 % in the presence of oligomycin. On the contrary, oligomycin activated the polyP synthesis in HEK cells. The same experiments have been then performed in isolated mitochondria with similar outcome, which suggests that mitochondria are in reality the place where polyP synthesis takes place. Such dependencies of polyP synthesis on the respiratory chain activity and oxidative phosphorylation, with involvement of the F_1F_0-ATP synthase, suggest that the production of polyP might be similar to ATP synthesis, using the proton gradient and pointing toward the ATP synthase as the main enzyme for polyP synthesis. Nonetheless, opposing effects of oligomycin on the level of polyP in different cells assume that there are at least several different ways of polyP synthesis in mammalian cell. Importantly, depletion of the polyP from the

mitochondria by expression of east PPX significantly alters mitochondrial metabolism, inhibits complex I-dependent respiration, and decreases mitochondrial membrane potential (Abramov et al. 2007).

Consequently, we have demonstrated that elimination of polyP from mitochondria, via insertion of east PPX into mitochondrial genome, increases mitochondrial Ca^{2+} capacity, lowers the susceptibility of these cells to PTP opening, and ultimately protects brain cells from β-amyloid peptide toxicity (Abramov et al. 2007). Increase of mitochondrial Ca^{2+} capacity and protection against cell death caused by application of β-amyloid peptide happened because of inability of mitochondria to open mitochondrial permeability transition (PT) pore in the absence of polyP. It is the second step of this process, suggesting that the presence of polyP is essential for PTP. Earlier on, Pavlov et al. isolated the mitochondrial complex consisting of polyP/Ca^{2+}/poly-β-hydroxybutyrate and showed that when incorporated into an artificial membrane, it mimics the properties of PTP (Pavlov et al. 2005). In this study the authors propose the hypothesis that this complex might be in fact the pore-forming complex of PTP. However, it is still unknown whether activation of PTP via Ca^{2+} or ROS shares the same pathway or target, despite the fact that in both cases PTP can be inhibited with cyclosporine A (CsA). Importantly, the role of polyP in mitochondrial permeability transition pore was confirmed in cardiac cells (Seidlmayer et al. 2012).

ATP is known to be a signaling molecule in the brain, which regulates multiple functions via stimulation of P2Y or P2X receptors (Gourine et al. 2005). Considering high concentration in the brain and relative structural similarity between polyP and ATP, we suggested that polyP may play a role in signal transduction.

We found that application of polyP of different length orthophosphate residues in the chain (14, 60, and 130), at physiological concentration, increases intracellular Ca^{2+} concentration for majority of astrocytes and small number of neurons (Holmstrom et al. 2013). Elimination of calcium from the extracellular buffer and in the presence of EGTA didn't prevent polyP-dependent Ca^{2+} release. However, emptying of the endoplasmic reticulum via blocking the SERCA by thapsigargin prevented polyP-dependent Ca^{2+} release. Application of a broad spectrum of specific inhibitors revealed that phospholipase C (PLC) is involved in this process. Activation of PLC leads to the cleavage of the membrane phospholipid PIP_2 into IP_3 and diacylglycerol (DAG). IP_3 reaches the ER where it binds with IP_3 receptors. This in turn leads to the opening of the IP_3-sensitive Ca^{2+} channels and release of Ca^{2+} from ER. Phospholipase C is generally a membrane-bound enzyme, which is activated via the α-subunit of a G protein. The involvement of purinergic receptors and more specifically P2Y1 receptors in this pathway was verified by application of specific inhibitors suramin (broad-spectrum P2 receptor antagonist) and MRS2279 (selective antagonists of $P2Y_1$ receptor), as well as the knockdown of the $P2RY_1$ gene via specific short hairpin RNA (shRNA). Thus, it became clear that the binding of polyP to the $P2Y_1$ receptors triggers activation of PLC, formation of IP_3, and consequently opening of Ca^{2+} channels on the ER and release of Ca^{2+} to the cytoplasm (Fig. 8.1).

Inorganic polyphosphate can be visualized in cells by using DAPI (4′,6-diamidino-2-phenylindole) and its polyP binding shift of the emission wavelength

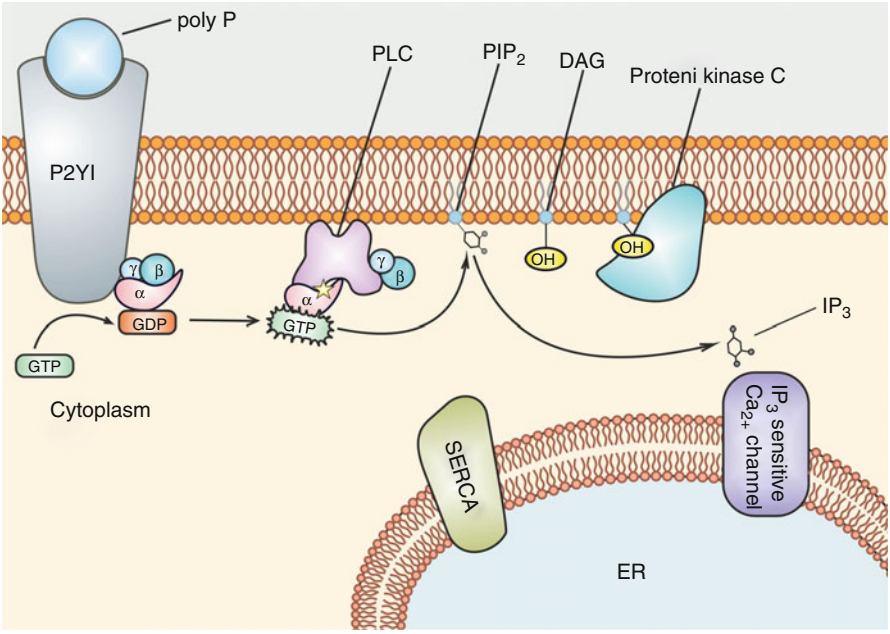

Fig. 8.1 Inorganic polyphosphate triggers IP_3 pathway of Ca^{2+} release from ER, via the $P2Y_1$ receptors

from 475 to 525 nm (Aschar-Sobbi et al. 2008). However, DAPI-polyP often binds to other polyanions within the cell, primarily to DNA and RNA. We have recently developed a highly specific fluorescent probe for visualization of polyP which exerts much higher affinity to polyP in contrast to ATP, RNA, and DNA (Angelova et al. 2014).

In astrocytes the distribution of the polyP signal reveals that it is present mainly in mitochondria, lysosomes, and cytoplasmic vesicle-like structures. Addition of exogenous polyP to cells loaded with polyP-sensitive fluorescent probe caused release of polyP from cytoplasmic vesicle-like structures. Interestingly, at the same time, polyP levels have increased in neighboring cells. Plasma membrane is impermeable for polyP; therefore, a drop in the polyP fluorescence would mean that neurons take polyP up from the presynaptic cleft via a mechanism that is still unclear. Application of Ca^{2+} ionophore – ionomycin – also initiated polyP release from astrocytes. To prove that polyP release from astrocytes has Ca^{2+}-dependent nature, we have used caged Ca^{2+} and flash photolysis to prompt an increase in Ca^{2+} concentration in a single cell. Excitation of one cell using Ca^{2+} uncaging technique led to an increase of Ca^{2+} concentration in neighboring non-flashed cells. Application of various inhibitors and ATP-degrading enzymes showed that specifically polyP is released from the flashed cells and triggers a rise of $[Ca^{2+}]_i$ in the neighboring cells through the $P2Y_1$ receptors (Holmstrom et al. 2013).

In our experiments, the presence of ATP-degrading enzymes, such as hexokinase and apyrase, during the experiments, had no effect on the numbers of responding cells and amplitudes of Ca^{2+} responses. Consequently, we can conclude that ATP does not participate in this particular Ca^{2+} response, but polyP does.

The function of polyP has been assessed also in in vivo experiments. Application of polyP to the chemosensory areas of the brainstem increased key cardiorespiratory characteristics such as breathing, central sympathetic outflow, heart rate, and arterial blood pressure. Application of different inhibitors of P2 receptors and specifically P2Y1 receptors did not affect the normal rate of these basic parameters but strongly decreased the effect of polyP (Holmstrom et al. 2013).

It is well known that polyP plays an important role in blood clotting in inflammation (Morrissey 2012). Recently it has been shown that one of the mechanisms of polyP proinflammatory effect relates to its binding with the $P2Y_1$ receptors on the plasma membrane of the endothelial cells and is mediated by the activation of IP_3 pathway of Ca^{2+} release from ER (Dinarvand et al. 2014) which proves the role of polyP in activation of purinergic signals.

Interesting report was made by Stotz et al. showing that inorganic polyphosphate fires an action potential in neurons through the potentiation of voltage-gated Na^+ and voltage-gated Ca^{2+} channel activity and partially through blocking the K_V channel activity (Stotz et al. 2014). It is well known that activation of calcium-dependent K^+ channels in hippocampal neurons is mediated by $P2Y_1$ receptors; however, suramin did not prevent polyP effect on K_V channels. The authors speculate that polyP does not bind to the channels directly but alters the net surface charge density of the membrane, which creates an environment favorable for Na_V and Ca_V channels to be activated. However, they do not exclude the possibility of direct interaction of polyP with the channels in the light of the recent findings concerning chaperone activity of polyP (Gray et al. 2014) and its direct ionic interactions with TRPM8 where it plays a role of possible structural component (Zakharian et al. 2009). Moreover, they have detected a large-scale polyP release from neurons into intercellular space during depolarization, where it could further stimulate neurons and astrocytes. In our experiments, we have shown that only 3% of neurons respond to exogenously applied polyP. However, it was reported that in their electrophysiological experiments, 100% of the tested neurons responded to polyP (Stotz et al. 2014). They have used a puffer pipette, which delivers polyP more closely to the target cells, which means that much more polyP locate near the channels in certain period of time.

Moreover, it was shown that polyP modulates the activities of TRPA1 and TRPM8 channels, suggesting that it may be involved in neuronal signaling (Kim and Cavanaugh 2007; Zakharian et al. 2009). It is well known that activation of excitable cells occurs through the timely opening and closing of Na^+, K^+, Ca^{2+}, and Cl^- channels as a response to various signals.

In conclusion, polyP plays an important role in the accurate functioning of mammalian cells and particularly in brain cells: polyP can be synthetized in brain cells;

however, the mammalian enzymes involved in this processes are still not unidentified, although polyP metabolism is closely associated with oxidative phosphorylation and F_1F_0-ATP synthase; polyP is an essential attribute to the PTP phenomenon; polyP can be involved in neurodegeneration related to the opening of the PTP (Alzheimer's, Parkinson's diseases, etc.); polyP acts as a gliotransmitter mediating communication between astrocytes through $P2Y_1$ receptors; polyP modulates the function of neuronal voltage-dependent Na^+, K^+, and Ca^{2+} channels.

References

Abramov AY, Fraley C, Diao CT et al (2007) Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death. Proc Natl Acad Sci U S A 104:18091–18096

Angelova PR, Agrawalla BK, Elustondo PA et al (2014) *In situ* investigation of mammalian inorganic polyphosphate localization using novel selective fluorescent probes JC-D7 and JC-D8. ACS Chem Biol 9:2101–2110

Aschar-Sobbi R, Abramov AY, Diao C et al (2008) High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. J Fluoresc 18:859–866

Dinarvand P, Hassanian SM, Qureshi SH et al (2014) Polyphosphate amplifies proinflammatory responses of nuclear proteins through interaction with receptor for advanced glycation end products and P2Y1 purinergic receptor. Blood 123:935–945

Gabel NW, Thomas V (1971) Evidence for the occurrence and distribution of inorganic polyphosphates in vertebrate tissues. J Neurochem 18:1229–1242

Gourine AV, Llaudet E, Dale N et al (2005) ATP is a mediator of chemosensory transduction in the central nervous system. Nature 436:108–111

Gray MJ, Wholey WY, Wagner NO et al (2014) Polyphosphate is a primordial chaperone. Mol Cell 53:689–699

Han KY, Hong BS, Yoon YJ et al (2007) Polyphosphate blocks tumour metastasis via anti-angiogenic activity. Biochem J 406:49–55

Hernandez-Ruiz L, Gonzalez-Garcia I, Castro C et al (2006) Inorganic polyphosphate and specific induction of apoptosis in human plasma cells. Haematologica 91:1180–1186

Holmstrom KM, Marina N, Baev AY et al (2013) Signalling properties of inorganic polyphosphate in the mammalian brain. Nat Commun 4:1362

Jimenez-Nunez MD, Moreno-Sanchez D, Hernandez-Ruiz L et al (2012) Myeloma cells contain high levels of inorganic polyphosphate which is associated with nucleolar transcription. Haematologica 97:1264–1271

Kawazoe Y, Shiba T, Nakamura R et al (2004) Induction of calcification in MC3T3-E1 cells by inorganic polyphosphate. J Dent Res 83:613–618

Kim D, Cavanaugh EJ (2007) Requirement of a soluble intracellular factor for activation of transient receptor potential A1 by pungent chemicals: role of inorganic polyphosphates. J Neurosci 27:6500–6509

Kumble KD, Kornberg A (1995) Inorganic polyphosphate in mammalian cells and tissues. J Biol Chem 270:5818–5822

Liebermann L (1888) Ueber das Nucleïn der Hefe und künstliche Darstellung eines Nucleïns aus Eiweiss und Metaphosphorsäure. Ber Chem Ges 21:598–607

Lorenz B, Munkner J, Oliveira MP et al (1997) Changes in metabolism of inorganic polyphosphate in rat tissues and human cells during development and apoptosis. Biochim Biophys Acta 1335:51–60

Morrissey JH (2012) Polyphosphate: a link between platelets, coagulation and inflammation. Int J Hematol 95:346–352

Pavlov E, Zakharian E, Bladen C et al (2005) A large, voltage-dependent channel, isolated from mitochondria by water-free chloroform extraction. Biophys J 88:2614–2625

Pavlov E, Aschar-Sobbi R, Campanella M et al (2010) Inorganic polyphosphate and energy metabolism in mammalian cells. J Biol Chem 285:9420–9428

Seidlmayer LK, Gomez-Garcia MR, Blatter LA et al (2012) Inorganic polyphosphate is a potent activator of the mitochondrial permeability transition pore in cardiac myocytes. J Gen Physiol 139:321–331

Stotz SC, Scott LO, Drummond-Main C et al (2014) Inorganic polyphosphate regulates neuronal excitability through modulation of voltage-gated channels. Mol Brain 7:42

Zakharian E, Thyagarajan B, French RJ et al (2009) Inorganic polyphosphate modulates TRPM8 channels. PLoS One 4:e5404

Inorganic Polyphosphate Functions and Metabolism in Insects

9

Fabio Mendonça Gomes, I.B. Ramos, H. Araujo, K. Miranda, and E.A. Ednildo

Abstract

Inorganic polyphosphates (polyP) are widespread molecules that are now known to be key cellular components and mediators of several metabolic pathways. Nevertheless, polyP metabolism among insects has remained relatively unexplored

F.M. Gomes (✉)
Laboratório de Bioquímica de Insetos e Parasitos (Labip), Instituto de Biofísica Carlos Chagas Filhos, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373 – bloco D, sala 13, Rio de Janeiro, RJ, Brazil

Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filhos, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
e-mail: fabiomg@biof.ufrj.br; fabio.mendoncagomes@nih.gov

I.B. Ramos
Laboratório de Bioquímica de Insetos, Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

H. Araujo
Laboratório de Biologia Molecular do Desenvolvimento, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular, Rio de Janeiro, Brazil

K. Miranda
Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filhos, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Centro Nacional de Biologia Estrutural e Bioimagem, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

E.A. Ednildo
Laboratório de Bioquímica de Insetos e Parasitos (Labip), Instituto de Biofísica Carlos Chagas Filhos, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373 – bloco D, sala 13, Rio de Janeiro, RJ, Brazil

Centro Nacional de Biologia Estrutural e Bioimagem, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

DIPRO-Diretoria de Programas, Instituto Nacional de Metrologia Normalização e Qualidade Industrial-RJ, Xerém, Duque de Caxias, Brazil

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_9

in comparison to bacterial and unicellular eukaryotic models. On the other side, aspects such as insect polyP storage and mobilization have provided interesting insights on how polyP is compartmentalized and regulated. These have later shown to present interesting parallels to mammalian and other chordate models. In the present report, polyP storage, mobilization, and its correlation with the regulation of phosphate and other free metal levels, as well as its implication for the regulation of insect homeostasis and development, are reviewed, and, whenever possible, a parallel is given to other eukaryotic models. We also point to some of the major open questions in the field, urging toward a further investigation of genes and enzymes regulating polyP metabolism.

9.1 Introduction

From being a forgotten cellular component once referred to as a "molecular fossil," inorganic polyphosphates (polyP) are now believed to be an essential constituent of every organism (Kornberg 1995) and have gained progressive attention from the scientific community. The identification of key polyP metabolic enzymes and disruption of polyP metabolism by genetic manipulation in bacteria and unicellular eukaryotes have shown that polyP play a major role in the survival of these organisms, notably by allowing adaptation to environmental stress (Rao et al. 2009). In eukaryotes, studies have shown that polyP is stored in different cell compartments, such as polyP-rich granules (commonly referred to as acidocalcisomes), cytoplasm, nucleus, mitochondria, cellular envelope, and plasma membrane (Rao et al. 2009), as well as physically associated with the structure of protein complexes, like membrane transporters (Zakharian et al. 2009; Stotz et al. 2014; Wei et al. 2015). Accordingly, a vast array of physiological functions for polyP among metazoans has been suggested, including the regulation of stem cell differentiation, bone formation, stress resilience, cell-to-cell communication, neuronal excitability, blood clot coagulation, and vascular permeability (Kawazoe et al. 2008; Tsutsumi et al. 2014; Stotz et al. 2014; Travers et al. 2015; Simbulan-Rosenthal et al. 2015). Strikingly, the metabolic pathways for polyP synthesis and degradation still remain mostly enigmatic in higher eukaryotes as no enzyme has been shown to undoubtedly drive polyP synthesis, and only a limited number of polyP-hydrolyzing enzymes have been described so far.

Among insects, polyP-related studies are still comparatively limited. Despite its amazing diversity and the socioeconomic impact of the interaction of several species with the human civilization, polyP-related studies are still restricted to a small number of insect models. Still, aspects such as polyP compartmentalization and store mobilization have seen an interesting growth of knowledge, not only contributing toward a better understanding of the roles of polyP among insects and other arthropods but also potentially providing a starting point for the investigation of other animal models. In the following sections, we have focused on the subcellular compartmentalization of polyP and its potential physiological roles among insect

models. It was our intent to describe areas where knowledge has been growing on a faster rate, as well as to point to relevant themes that remain obscure and have hindered further progress on the field.

9.2 PolyP Storage in Insect Oocytes and Eggs

Insects are oviparous animals, and their eggs are usually laid in a terrestrial environment protected by an impermeable sheath where the embryo development occurs isolated from the environment and maternal body. Thereby, all the nutrients and energy sources required for the growth, division, and differentiation of the embryo cells are previously stored inside the oocytes during oogenesis in the maternal ovary. It is believed that most of the synthesis of these storage components is performed by the fat body – a biosynthetic organ functionally similar to the human liver (Raikhel and Dhadialla 1992; Snigirevskaya et al. 1997). There, the proteins and other macromolecules are synthesized and secreted into the hemolymph (circulation fluid of the open circulatory system found in insects). Those macromolecules are incorporated into oocytes by receptor-mediated endocytosis and stored within the cytoplasm into different endocytic organelles collectively called yolk granules (YG). At the end of oogenesis, an insect oocyte can grow up to 4,000 times its original size, and most of the cytoplasm volume will be filled with YG.

In most insect species, vitellin (VT) is the main yolk protein synthesized by the fat body and accumulated into YG. It is believed that, as a phospholipidglycoprotein, VT provides the embryo cells with amino acids, phosphates, lipids, and sugars upon its mobilization. From a cell biology point of view, the YG have been compared to latent lysosomes. Hydrolytic activities are usually carried out by classic lysosomal enzymes regulated by proton-pump-dependent YG acidification, as usually observed during early embryogenesis of different species (Fagotto 1995). In this regard, modulation of the proton-pump activity allows the degradation of yolk macromolecules in a pace that meets the anabolic demand of the developing embryo and prevents early or excessive yolk mobilization. The presence of such regulatory mechanisms is reflected by the fact that VT hydrolysis and proteolytic activity are usually undetectable at the early steps of embryo development and are activated after the onset of gastrulation. Unfortunately, the molecular and cellular mechanisms underlying this regulated hydrolysis have remained elusive.

Traditionally, the literature refers to all of the oocyte/egg endocytic organelles as YG, but it has been shown that this comprises a heterogeneous population of organelles with distinct morphology and content, raising the question of whether phosphate and polyP-specialized stores could be identified among insect eggs. Accordingly, investigations on polyP storage using fluorescent probes led to the identification of a subset of distinguishable small vesicles containing high amounts of polyP in the eggs of the American cockroach *Periplaneta americana* and the kissing bug *Rhodnius prolixus* (Motta et al. 2009; Ramos et al. 2011). In addition to their small size (~500 nm), these polyP-rich granules also distinguished from the

typical larger YG (1 to up to 100 μm) by their elemental content composed mainly by phosphorous and metallic atoms like calcium, iron, and magnesium (Fig. 9.1). This characteristic elemental profile is similar to a family of well-known polyP-rich granules previously named acidocalcisomes. Acidocalcisomes have been described in several models so far and have been proposed to be highly conserved during

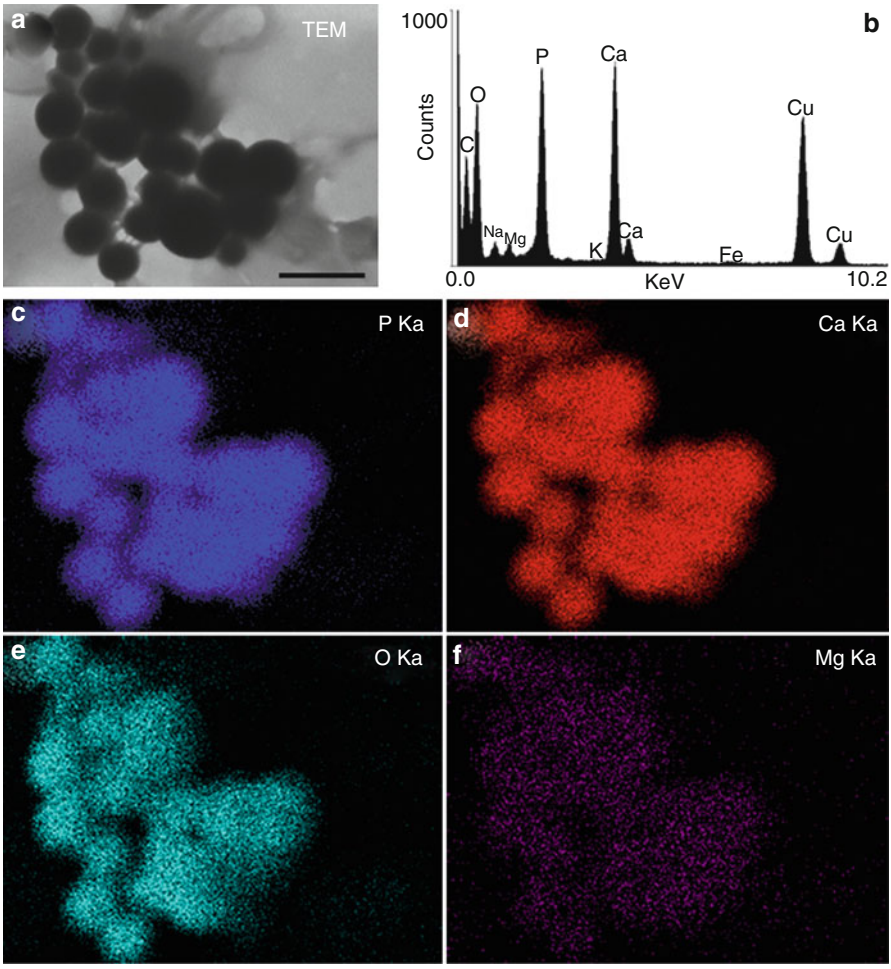

Fig. 9.1 Acidocalcisome-like structures are found among insect eggs. As exemplified by *R. prolixus*, acidocalcisome-like structures are apparently widespread among insect eggs. (**a**) These structures store vast amounts of polyP and can be differentiated from the typical YG by their high electron density under the transmission electron microscope. (**b**) Analytical microscopy analysis reveals an elemental profile similar to the one described for acidocalcisomes from other cell types, characterized by significant amounts of phosphorus and metallic atoms, such as calcium, sodium, and magnesium, and low or undetectable levels of sulfur (denoting relatively low levels of stored proteins). (**c**–**e**) Elemental mapping illustrates the homogenous atomic composition of these structures as exemplified by the distribution of *P* phosphorus, *Ca* calcium, *O* oxygen, and *Mg* magnesium. *Bar*: 1 μm

evolution (Docampo et al. 2005). Indeed, similar structures have later been described in sea urchin and chicken eggs (Ramos et al. 2010). The acidocalcisome-like organelles found in the cytoplasm of insect eggs have all the morphological and content features displayed by the canonical acidocalcisomes: high electron density content when observed under the transmission electron microscopy (TEM), high levels of polyP, and different elements like phosphorus (from polyP, PP_i, and P_i), sodium, magnesium, calcium, iron, and chloride. It is known that the regulation of acidocalcisome function is strongly associated with its luminal acidification (Docampo et al. 2005). Accordingly, insect polyP-rich acidocalcisome-like vesicles were also shown to be acidified. Interestingly, this acidification was more frequently observed a few days after fertilization/oviposition (typically after intracellular cleavage, at the onset of gastrulation), suggesting a regulatory mechanism of luminal acidification. Acidocalcisome-like structures are also found in *D. melanogaster* eggs (Fig. 9.2a, b) where polyP is concentrated at the egg cortex (Fig. 9.2c, d). This polyP distribution is similar to acidocalcisome-like distribution described in *R. prolixus* eggs (Ramos et al. 2011). An interesting hypothesis is that local signals originate from polyP-rich stores and interact with the embryo cells during cellularization and blastoderm formation.

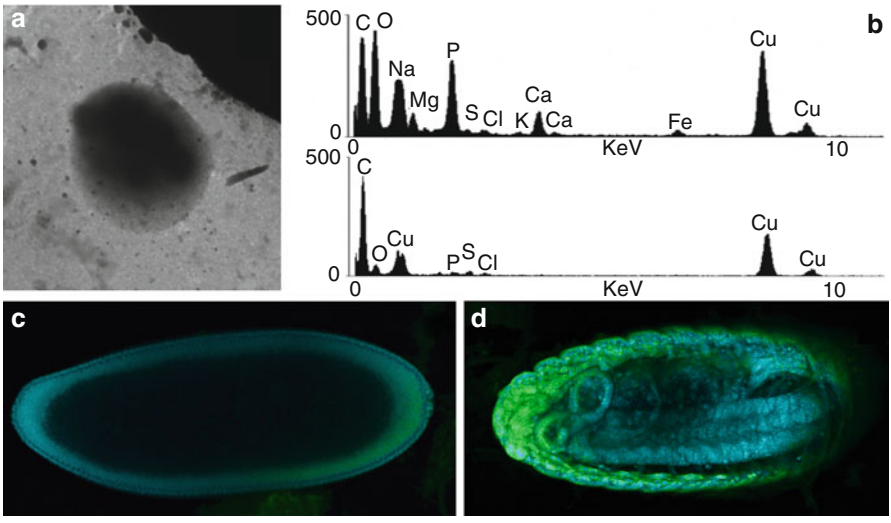

Fig. 9.2 (**a**) Electron-dense polyP-rich acidocalcisome-like structures are also identified in egg homogenates from *D. melanogaster* and (**b**, *top half*) present a similar elemental profile as those identified in *R. prolixus* (**b**, *bottom half*), which is distinct from the elemental composition of surrounding regions of the microscope grid. By the yellowish emission of DAPI fluorescence (525–550 nm) when bound to polyP, polyP-enriched regions can be identified in the cortex of *Drosophila* embryos. (**c**) Optical cross section of a syncytial blastoderm stage embryo. Staining is observed in the cortical cytoplasm surrounding the blastoderm nuclei. (**d**) Optical cross section of a stage 15 embryo. Staining is seen in the superficial muscular layer. No staining is observed in the nerve cord. In both (**c**) and (**d**), anterior is oriented at the left of the figure

9.2.1 Acidocalcisome-Like PolyP Storage, P_i Reservoir, and Cation Sequestration

Acidocalcisome-like organelles in oocytes and eggs store an important fraction of the total P_i and metal content available for the developing embryo. For example, isolation of these vesicles from *R. prolixus* oocytes and eggs has shown that these organelles store approximately 25 % of the total amount of calcium detected in the egg. More striking, polyP from polyP-rich vesicles accounts for more than 80 % of the total egg polyP in *R. prolixus* (Ramos et al. 2011). The fact that polyP functions as a P_i store in several models (Kornberg et al. 1999; Kulaev and Kulakovskaya 2000) has motivated an initial investigation on whether or not similar roles could be found during insect development. This was first investigated during the development of *P. americana* (Gomes et al. 2008). Using DAPI as a fluorescent probe, polyP mobilization was analyzed at the early days of embryo development. Here, levels of polyP and P_i were maximal at the time of oviposition, and there was a continuous decrease of the levels of both components during the first 5 days of development. It was hypothesized that anabolic reactions during *P. americana* embryo development, such as the massive synthesis of DNA, could act as a P_i sink and drive polyP mobilization as a compensatory mechanism. This is similar to the regulation of P_i homeostasis in S*accharomyces cerevisiae* by the phosphate signal transduction (PHO) pathway where vacuolar polyP mobilization is activated when cytoplasmic P_i reaches a critical level (Shirahama et al. 1996; Secco et al. 2012) and had also a similar profile to what was later shown during the embryogenesis of the phylogenetically close arthropod *Rhipicephalus microplus* (Campos et al. 2008). Interestingly, polyP and P_i correlation showed a different scenario in the kissing bug *R. prolixus* with polyP hydrolysis also preceding the onset of VT mobilization but accompanied by a concomitant increase of P_i levels (Fig. 9.3a). This raises the question of whether the reduction of P_i levels during *P. americana* development might be triggering polyP mobilization or simply reflecting the balance between P_i consumption and release. Altogether, the data points to the fact that a progressive polyP mobilization takes place during early development (before the onset of VT mobilization). More interestingly, the widespread correlation between polyP and P_i levels at this period supports the hypothesis that polyP stores are used as a P_i source during early embryo development, placing polyP as an essential yolk macromolecule.

Another attention-grabbing property of polyP that seems to be explored by several models and cell types is its potential function as cation chelator and osmolarity regulator. PolyP negative charges account for the ability of this polymer to bind and chelate free cations (Kornberg et al. 1999; Kulaev and Kulakovskaya 2000). In this regard, regulated synthesis and degradation of polyP coupled with the function of ion pumps, ionophores, and exchangers in the vesicle membrane represent a compartmentalized system that is able to rapidly regulate Ca^{2+} and possibly other cations (Docampo et al. 2013). PolyP mobilization during early embryogenesis also accounted for a regulation step toward availability of metals in the oocyte cytoplasm. Not only polyP was found mainly stored in calcium-rich compartments (as described in above sections), but the levels of free Ca^{2+} in egg homogenates

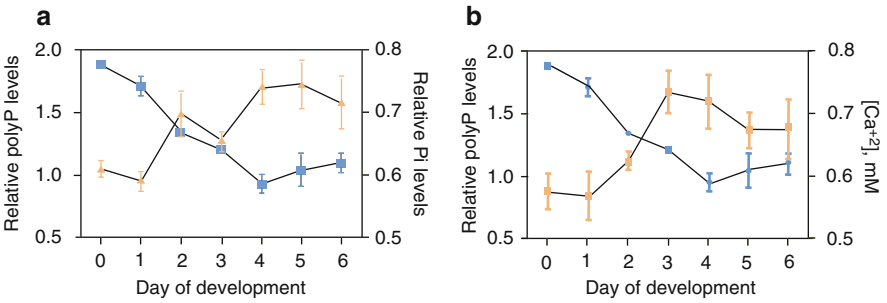

Fig. 9.3 PolyP, P_i, and Ca^{2+} mobilization of polyP during early embryogenesis. As illustrated by the levels of polyP in egg homogenates from *R. prolixus*, total polyP content (*blue line*) is mobilized during the early steps of embryo development and halted by the moment that VT mobilization starts (at days 3 and 4 in *R. prolixus*). PolyP mobilization is correlated with (**a**, *orange line*) Pi and (**b**, *orange line*) Ca^{2+} mobilization at the same period, suggesting at co-regulation of the levels of these three components

correlated with polyP levels in both *P. americana* (Gomes et al. 2008) and *R. prolixus* (Fig. 9.3b), where the decrease of polyP levels matched the increase of free Ca^{2+} levels in the ooplasm.

In unicellular eukaryotes, the inhibition of the vacuolar H^+-ATPase of acidocalcisomes decreases proton uptake, and, due to the action of a number of H^+/cation countertransporting systems, the organelle is alkalinized. This alkalization results in polyP hydrolysis and Ca^{2+} release through a Ca^{2+}/H^+ exchanger. Thus, calcium uptake is thought to be driven by the proton gradient, which is coupled to a Ca^{2+}/H^+ countertransporting ATPase (Docampo et al. 2005). Although the detailed mechanisms are not well understood, it is known that acidocalcisomes are able to release calcium in some cell types via IP3 receptor (Huang et al. 2013). Whether similar mechanisms take place in insect acidocalcisome-like compartments is a matter of further studies.

9.2.2 Interplay Between PolyP and Yolk Mobilization

As mentioned before, yolk mobilization must be regulated in order to match the embryo nutritional demand during development, but the mechanisms underlying this regulation are mostly unknown. In *R. prolixus*, it has been shown that an aspartic protease cathepsin D (CD) is the major protease involved with VT hydrolysis during embryo development (Nussenzveig et al. 1992). Strikingly, the inhibition of YG acid phosphatase (AP) also induces a similar inhibition of VT hydrolysis, showing that the activation of yolk mobilization by CD also depends on AP activation (Fialho et al. 2005). By the time the eggs are laid, AP seems to be stored in a restricted subset of vesicles apart from the VT-rich YG (Ramos et al. 2007). Later at development, co-localization of AP, CD, and VT is modulated by Ca^{2+}-mediated fusion events (Ramos et al. 2007). Thus, a hypothetical model could be set where CD and/or VT are spatially separated in different compartments in developing oocytes and fresh

laid eggs. After fertilization, regulated fusion of different organelles would bring co-localized AP and CD inside VT-rich YG, allowing VT mobilization to proceed.

Three lines of evidence suggested that polyP could be the physiological substrate of AP following vesicle fusion. First, DAPI fluorescence revealed that polyP is stored inside the YG of *R. prolixus* and *P. americana* (in lower concentrations than in the acidocalcisome-like vesicles). Second, phosphatases from several models have been shown to hydrolyze polyP of different chain lengths. For example, calf digestive alkaline phosphatase presents exopolyphosphatase activity, which was suggested to account for polyP mobilization during food digestion (Lorenz and Schroder 2001). Third, polyP has been shown to modulate the activity of mammalian cathepsin D (Watabe et al. 1979, 1996; Ducastaing et al. 1976), suggesting that it could also modulate CD. Hypothesis testing was performed by adding polyP to a set of assays using *R. prolixus* egg homogenates (Gomes et al. 2010). In that sense, the addition of polyP (>15 P_i residues) was able to block in vitro CD activity from *R. prolixus* eggs and to halt CD-mediated yolk protein mobilization in egg homogenates. At the same time, polyP was shown to be processed in vitro by AP. Finally, when vesicle fusion was induced in vitro by adding Ca^{2+} to vesicle suspensions, the ability of YG polyP fractions to inhibit CD activity was strongly diminished (supposedly as a result of AP-mediated hydrolysis of polyP). Together this suggests a model where polyP is stored inside YG in freshly laid eggs and functions toward arresting CD activation and, thus, yolk mobilization. Ca^{2+} signals during early development could induce regulated vesicle fusion and delivery of AP to the YG, driving the hydrolysis of the YG pool of polyP and allowing CD activation (Fig. 9.4 – Steps 3, 4). Additionally, as the raise of the cytoplasmic Ca^{2+} levels correlates with the rate of mobilization of total egg polyP, an attractive extension of this working model is that mobilization of polyP from acidocalcisome-like organelles during early embryogenesis might release Ca^{2+} and induce vesicle fusion (Fig. 9.4 – Steps 1, 2).

Although not as extensively investigated, a similar interplay between polyP and yolk proteases was described in the eggs of *A. gemmatalis* (Oliveira et al. 2013). In this model, a cysteine protease seems to be the major protease present in YG, and polyP was shown to also inhibit its activity. Different from *R. prolixus*, *A. gemmatalis* protease activity was preferentially inhibited by short-chain polyP. Accordingly, acid phosphatase from *A. gemmatalis* has a preference for short-chain polyP residues. Similar to *R. prolixus*, acid phosphatase activity in freshly laid eggs was also found in small vesicles apart from the major YG, suggesting that fusion events might also regulate yolk mobilization in *A. gemmatalis*. Together, this points to a conserved role of polyP toward regulation of VT mobilization among insect models. At the same time, it opens the question of how polyP would be able to regulate evolutionary unrelated enzymes.

Additionally, VT mobilization depends on the activation of the proton pumps that modulate YG acidification. How this is accomplished is also a matter of discussion, but evidences suggest that Ca^{2+}-mediated vesicle fusion is also linked with the transfer of acidocalcisome-like proton pumps to YG membrane (Ramos et al. 2007). Thus, the regulation of the acidification potential of YG is another interesting putative role of polyP-rich organelles.

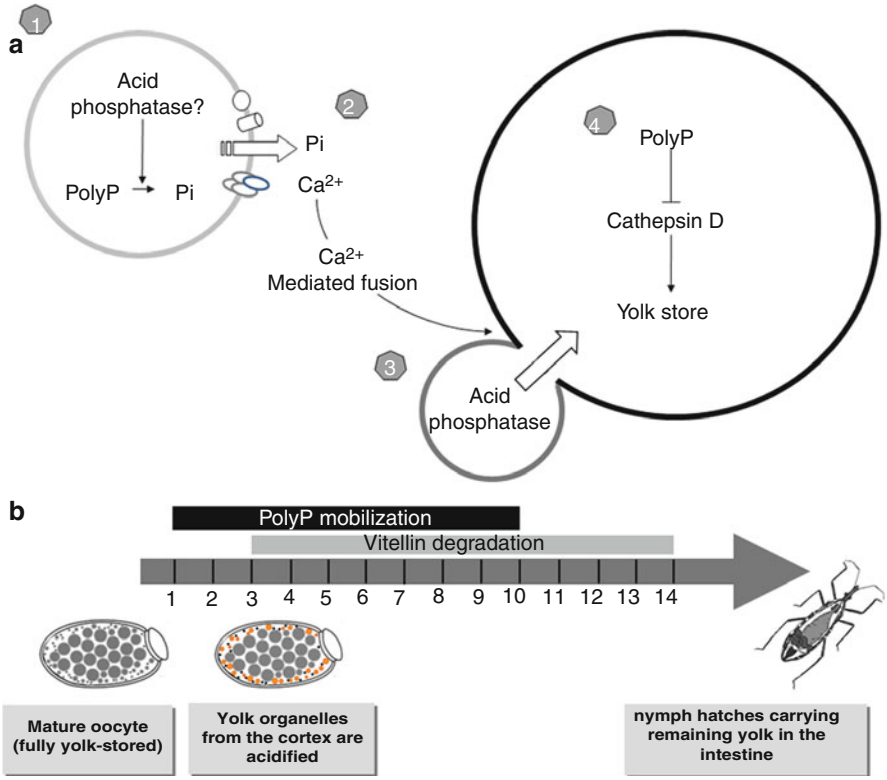

Fig. 9.4 PolyP-mediated yolk mobilization of *R. prolixus*. Published data from *R. prolixus* infer that VT mobilization is regulated at different steps and driven by a succession of events where polyP plays a central role. (**a**) Step 1: Acidocalcisome-like polyP pool is hydrolyzed by an unknown effector enzyme (possibly an acid phosphatase). Step 2: polyP mobilization drives the increase of Ca^{2+} and P_i levels in the cytoplasm. Step 3: Increase of cytoplasmic levels of Ca^{2+} triggers fusion events between acid phosphatase-containing organelles and YG – it is not clear whether acid phosphatase stores and acidocalcisome-like organelles are distinct or identical compartments. Step 4: Compartmentalization of acid phosphatases inside YG mobilizes YG polyP store that restrained CD activity. PolyP mobilization allows CD-mediated VT proteolysis to proceed. (**b**) Thus, polyP mobilization is an event that precedes and regulates VT degradation and is timely correlated with the acidification of organelles in the cortex of the eggs

9.2.3 PolyP Stores in Mitochondria and Other Cellular Compartments of Oocytes and Eggs

As mentioned, polyP is found to be stored in the mitochondria of different models and has been related to different functions, including the regulation of energetic metabolism, opening of the permeability transition pore, and regulation of cell death (Abramov et al. 2007; Pavlov et al. 2010; Seidlmayer et al. 2012). Nuclear polyP storage has also been described. Nucleolar polyP has been suggested to regulate RNA polymerase I activity, and a link between abnormally high-nucleolar polyP

storage and myeloma phenotypes has been presented (Jimenez-Nunez et al. 2012). Additionally, earlier studies have suggested that polyP is able to bind to nonhistone nuclear proteins and chromatin (Mansurova et al. 1975; Weinstein and Li 1976; Offenbacher and Kline 1984), but physiological relevance of these data is yet to be elucidated.

Studies using eggs from the tick *R. microplus* have detected the presence of polyP stored in mitochondrial and nuclei-enriched fractions (Campos et al. 2008). Taking into account the close evolutionary relationship between arachnids and insects, this data might mirror physiological roles performed by polyP during insect development. In *R. microplus*, polyP mobilization from mitochondria-enriched fractions was shown to correlate with the increase of embryo energetic demand (Campos et al. 2008). Mitochondrial polyP mobilization profile did not correlate with total polyP and nuclei-enriched polyP mobilization at the same period, evidencing that different polyP pools are under the control of independent pathways. Interestingly, in vitro adenosine diphosphate (ADP)-dependent mitochondrial oxygen consumption was detected when polyP was used as a P_i source, with a preference for short-chain polyP, suggesting that polyP can be used as phosphate donor for adenosine triphosphate (ATP) synthesis. This is similar to the description of cross talk between polyP and ATP levels in mammalian models (Abramov et al. 2007; Pavlov et al. 2010), pointing to a conserved role of polyP toward regulation of energetic metabolism among metazoans.

9.3 PolyP Storage in Spherites in the Midgut of Insects

The identification of polyP-rich organelles in insect eggs has raised the question of whether or not similar organelles are also present in tissues and cells at later periods in the life span of insects. The gut of insects and other invertebrates is an interesting model of metal homeostasis as the metal composition of epithelial cells of the midgut usually reflects the metal composition of ingested food and surrounding environment (Ballan-Dufrancais 2002). At the subcellular level, gut metal sequestration is usually performed by organelles known as spherites (Turbeck 1974; Humbert 1978; Da Cruz-Landim and Serrão 1996). Interestingly, spherite elemental composition presents a strong similarity with the egg acidocalcisome-like organelles, suggesting that polyP pools could also be detected inside spherites and might play a role toward metal buffering in this tissue. Accordingly, initial studies evidenced that spherites from *A. gemmatalis* present the typical acidocalcisome-like elemental profile and store high levels of polyP (Gomes et al. 2012). Additionally, the modulation of the diet composition by dietary copper supplementation resulted in copper uptake into spherites and correlated with polyP accumulation as detected by enzymatic and analytical microscopy assays.

In polyP-rich granules from other models, an array of transporters has been shown to modulate metal homeostasis (Docampo and Moreno 2011; Huang and Docampo 2015). When tested, drugs targeting acidocalcisome transporters affected the elemental profile of *Anticarsia* spherites (Gomes et al. 2012), evidencing that

functional similarities between these organelles are present. More specifically, sodium orthovanadate and bafilomycin A1 – known inhibitors of the acidocalcisome Ca^{2+}-ATPase and V-H^{+}-ATPase, respectively – induced the decrease of spherites calcium and zinc levels.

This raises the question of whether metal sequestration in polyP-rich granules is a more widespread feature and how this relates to specific challenges faced by different cell types in different insects. For example, blood-feeding insects have adapted to respond against the ingestion of massive amount of iron following a blood meal, as a result of hemoglobin digestion and intake of free iron present in the blood. Iron storage in polyP-rich granules might play a role toward minimizing toxic effects (e.g., oxidative stress) that would incur from excessive availability of free iron. Similarly, Malpighian tubule concretion bodies present morphological and functional similarities to the midgut spherites (Ballan-Dufrancais 2002; Talarico et al. 2014) and might also use polyP as a cation chelator agent.

9.4 Enzymes of PolyP Metabolism in Insects

In bacteria, polyP synthesis is driven by two unrelated groups of polyP kinases (PPK) named PPK1 and PPK2 that transfer terminal Pi from ATP into either polyP or PP_i (Ahn and Kornberg 1990; Zhang et al. 2002). Both enzymes synthesize long polyP polymers that are later processed by different classes of enzymes. Exopolyphosphatases (PPX) are the most prominent group of polyP-mobilizing enzymes and are responsible for progressive removal of terminal Pi from polyP polymers (Akiyama et al. 1993). Additionally, a set of kinases have been shown to be able to incorporate polyP as P_i donors along different metabolic pathways (Hsieh et al. 1996; Shiba et al. 2005; Lindner et al. 2010). The major known enzymes of polyP metabolism in eukaryotes do not share homologies with the bacterial enzymes, raising questions about the evolution of polyP-related metabolic pathways. In yeasts, a protein complex found at the membrane of polyP-rich granules (vacuolar transporter chaperone – VTC) is responsible for the synthesis of most of the cellular polyP (Hothorn et al. 2009). Accordingly, data from protozoans suggest that VTC-derived polyP synthesis also accounts for PolyP storage in acidocalcisome-like compartments (Fang et al. 2007; Lander et al. 2013; Ulrich et al. 2014). PolyP mobilization is performed by PPX-like enzymes containing a DHH-conserved motif in their linear structure (Aravind and Koonin 1998) or by endopolyphosphatases (PPN) that are able to hydrolyze internal bonds of polyP (Shi and Kornberg 2005). Additionally, both PPN and soluble pyrophosphatases have been shown to present PPX activity (Espiau et al. 2006; Andreeva et al. 2015).

Compared to bacteria and unicellular eukaryotes, polyP metabolic pathways in metazoans are less well understood. No clear orthologues of either PPK or VTC have been found, and it is not clear how polyP is synthesized by those organisms. On the other side, a DHH motif-containing enzyme has been shown to have exopolyphosphatase activity in humans (Tammenkoski et al. 2008). PPN activity has

also been described in mammalian cells (Kumble and Kornberg 1996), but its genetic origin remains to be determined. In arthropods, PPX activity has been demonstrated in mitochondria-enriched fractions of *R. microplus* (Campos et al. 2008), and a link with energetic metabolism has been provided (see above sections). Nevertheless, the gene associated with this activity was not identified. DHH-containing homologs are found among insect genomes (Fig. 9.5), but it is not clear that such enzymes account for mitochondrial polyP mobilization. Interestingly, silencing of a soluble pyrophosphatase in the eggs of the red flour beetle *Tribolium castaneum* resulted in a decrease of PPX activity, suggesting that pyrophosphatases might also have polyP-processing roles in insects (Carvalho et al. 2015). As in other models, phosphatases are putative polyP-processing enzymes, but physiological roles apart from the case of *R. prolixus* eggs (see above) are yet to be established. One interesting open question refers to what extent phosphotransferases are able to use polyP as a phosphate donor. Opposing to bacteria, hexokinases from *R. microplus* were inhibited by polyP suggesting that polyP is not used as a hexokinase substrate (Fraga et al. 2013).

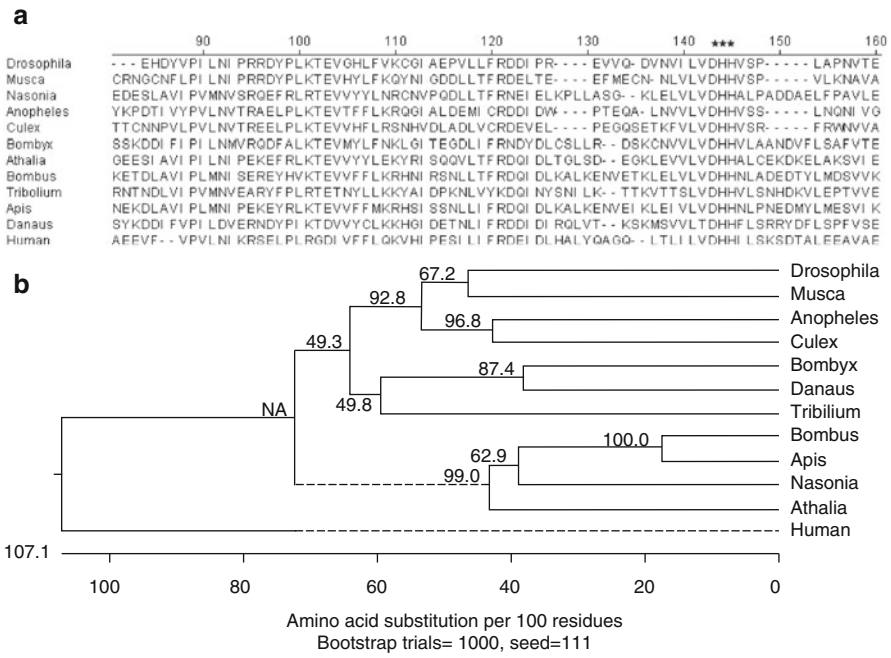

Fig. 9.5 Ubiquitous presence of exopolyphosphatase candidates among insect genomes. (**a**) *D. melanogaster* prune homologs can be identified among several insect genomes suggesting putative candidates for exopolyphosphatase-like enzymes as can be shown by the alignment of the region flanking the identified DHH motif signature (identified by *asterisks*). (**b**) Phylogenetic tree of the identified sequences. *Drosophila*, *D. melanogaster*; *Musca*, *Musca domestica*; *Nasonia*, *Nasonia vitripennis*; *Anopheles*, *Anopheles gambiae*; *Culex*, *Culex quinquefasciatus*; *Bombyx*, *Bombyx mori*; *Athalia*, *Athalia rosae*; *Bombus*, *Bombus terrestris*; *Tribolium*, *Tribolium castaneum*; *Apis*, *Apis mellifera*; *Danaus*, *Danaus plexippus*

9.5 Concluding Remarks and Future Directions

At present, most polyP-related studies among insects have focused on understanding how cellular polyP is stored and modulated. This provides an interesting case of study, and further comparative investigations of vertebrate models are likely to raise interesting insights on how these compartments have been conserved during metazoan evolution. Description of this organelle by proteomic analysis followed by comparisons among different models will allow a better understanding of the evolution of polyP metabolism, as well as the identification of transporters and receptors involved with metal uptake. polyP stored in human platelet dense granules is released during platelet degranulation (Ruiz et al. 2004). Similarly, polyP has been identified inside apocrine secretion release from midgut enterocytes of *A. gemmatalis* (Gomes et al. 2013), indicating a similar route of polyP secretion that might have physiological significance to coordinate organism homeostasis.

In this regard, some of the challenges faced by entomologists are similar to those from the ones studying polyP metabolism in other metazoans, e.g., identification of key polyP-metabolizing enzymes. Utilization of models such as *D. melanogaster* and derived cell culture lines might represent an invaluable tool for high-throughput assays, as was the case with lower eukaryotes (Ogawa et al. 2000). In that sense, identification of the genes involved with polyP synthesis should become a keystone step toward development of functional genomic assays and a deeper insight into the physiological roles performed by polyP.

References

Abramov AY, Fraley C, Diao CT et al (2007) Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death. Proc Natl Acad Sci U S A 104(46):18091–18096. doi:10.1073/pnas.0708959104

Ahn K, Kornberg A (1990) Polyphosphate kinase from *Escherichia coli*. Purification and demonstration of a phosphoenzyme intermediate. J Biol Chem 265(20):11734–11739

Akiyama M, Crooke E, Kornberg A (1993) An exopolyphosphatase of *Escherichia coli*. The enzyme and its ppx gene in a polyphosphate operon. J Biol Chem 268(1):633–639

Andreeva N, Trilisenko L, Eldarov M et al (2015) Polyphosphatase PPN1 of *Saccharomyces cerevisiae*: switching of exopolyphosphatase and endopolyphosphatase activities. PLoS One 10(3):e0119594. doi:10.1371/journal.pone.0119594

Aravind L, Koonin EV (1998) A novel family of predicted phosphodiesterases includes *Drosophila* prune protein and bacterial RecJ exonuclease. Trends Biochem Sci 23(1):17–19

Ballan-Dufrancais C (2002) Localization of metals in cells of pterygote insects. Microsc Res Technol 56(6):403–420. doi:10.1002/jemt.10041

Campos E, Facanha AR, Costa EP, Da Silva VI Jr, Masuda A, Logullo C (2008) Exopolyphosphatases in nuclear and mitochondrial fractions during embryogenesis of the hard tick *Rhipicephalus (Boophilus) microplus*. Comp Biochem Physiol B Biochem Mol Biol 151(3):311–316. doi:10.1016/j.cbpb.2008.07.013

Carvalho K, Ribeiro L, Moraes J et al (2015) A soluble pyrophosphatase is essential to oogenesis and is required for polyphosphate metabolism in the red flour beetle (*Tribolium castaneum*). Int J Mol Sci 16(4):6631–6644. doi:10.3390/ijms16046631

Da Cruz-Landim C, Serrão JE (1996) Ultrastructure and histochemistry of the mineral concretions in the midgut of bees (Hymenoptera: Apidae). Neth J Zool 47(1):21–29

Docampo R, Moreno SN (2011) Acidocalcisomes. Cell Calcium 50(2):113–119. doi:10.1016/j.ceca.2011.05.012

Docampo R, Jimenez V, Lander N et al (2013) New insights into the roles of acidocalcisomes and the Contractile Vacuole Complex in osmoregulation in Protists. Int Rev Cell Mol Biol 305:69–113. doi:10.1016/B978-0-12-407695-2.00002-0

Docamppoly P (2005) Stored in platelet dense granules is releasedo, Roberto, et al. "Acidocalcisomes? Conserved from bacteria to man." Nature Reviews Microbiology 3.3:251–261

Ducastaing A, Azanza JL, Raymond J et al (1976) Cathepsin D from horse spleen. II. Study of certain enzymatic properties. Biochimie 58(7):783–791

Espiau B, Lemercier G, Ambit A et al (2006) A soluble pyrophosphatase, a key enzyme for polyphosphate metabolism in *Leishmania*. J Biol Chem 281(3):1516–1523. doi:10.1074/jbc.M506947200

Fagotto F (1995) Regulation of yolk degradation, or how to make sleepy lysosomes. J Cell Sci 108(Pt 12):3645–3647

Fang J, Rohloff P, Miranda K et al (2007) Ablation of a small transmembrane protein of *Trypanosoma brucei* (TbVTC1) involved in the synthesis of polyphosphate alters acidocalcisome biogenesis and function, and leads to a cytokinesis defect. Biochem J 407(2):161–170. doi:10.1042/BJ20070612

Fialho E, Nakamura A, Juliano L et al (2005) Cathepsin D-mediated yolk protein degradation is blocked by acid phosphatase inhibitors. Arch Biochem Biophys 436(2):246–253. doi:10.1016/j.abb.2005.01.005

Fraga A, Moraes J, da Silva JR et al (2013) Inorganic polyphosphates regulate hexokinase activity and reactive oxygen species generation in mitochondria of *Rhipicephalus (Boophilus) microplus* embryo. Int J Biol Sci 9(8):842–852. doi:10.7150/ijbs.6628

Gomes FM, Ramos IB, Motta LM et al (2008) Polyphosphate polymers during early embryogenesis of *Periplaneta americana*. J Insect Physiol 54(12):1459–1466. doi:10.1016/j.jinsphys.2008.07.018

Gomes FM, Oliveira DM, Motta LS et al (2010) Inorganic polyphosphate inhibits an aspartic protease-like activity in the eggs of *Rhodnius prolixus* (Stahl) and impairs yolk mobilization in vitro. J Cell Physiol 222(3):606–611. doi:10.1002/jcp.21975

Gomes FM, Carvalho DB, Peron AC et al (2012) Inorganic polyphosphates are stored in spherites within the midgut of *Anticarsia gemmatalis* and play a role in copper detoxification. J Insect Physiol 58(2):211–219. doi:10.1016/j.jinsphys.2011.09.008

Gomes FM, Carvalho DB, Machado EA et al (2013) Ultrastructural and functional analysis of secretory goblet cells in the midgut of the lepidopteran *Anticarsia gemmatalis*. Cell Tissue Res 352(2):313–326. doi:10.1007/s00441-013-1563-4

Hothorn M, Neumann H, Lenherr ED et al (2009) Catalytic core of a membrane-associated eukaryotic polyphosphate polymerase. Science 324(5926):513–516. doi:10.1126/science.1168120

Hsieh PC, Shenoy BC, Samols D et al (1996) Cloning, expression, and characterization of polyphosphate glucokinase from *Mycobacterium tuberculosis*. J Biol Chem 271(9):4909–4915

Huang G, Docampo R (2015) Proteomic analysis of acidocalcisomes of *Trypanosoma brucei* uncovers their role in phosphate metabolism, cation homeostasis, and calcium signaling. Commun Integr Biol 8(2):e1017174

Huang G, Bartlett PJ, Thomas AP et al (2013) Acidocalcisomes of *Trypanosoma brucei* have an inositol 1, 4, 5-trisphosphate receptor that is required for growth and infectivity. Proc Natl Acad Sci U S A 110(5):1887–1892

Humbert W (1978) Cytochemistry and x-ray microprobe analysis of the midgut of *Tomocerus minor lubbock* (insecta, collembola) with special reference to the physiological significance of the mineral concretions. Cell Tissue Res 187(3):397–416

Jimenez-Nunez MD, Moreno-Sanchez D, Hernandez-Ruiz L et al (2012) Myeloma cells contain high levels of inorganic polyphosphate which is associated with nucleolar transcription. Haematologica 97(8):1264–1271. doi:10.3324/haematol.2011.051409

Kawazoe Y, Katoh S, Onodera Y et al (2008) Activation of the FGF signaling pathway and subsequent induction of mesenchymal stem cell differentiation by inorganic polyphosphate. Int J Biol Sci 4(1):37–47

Kornberg A (1995) Inorganic polyphosphate: toward making a forgotten polymer unforgettable. J Bacteriol 177(3):491–496

Kornberg A, Rao NN, Ault-Riche D (1999) Inorganic polyphosphate: a molecule of many functions. Ann Rev Biochem 68:89–125. doi:10.1146/annurev.biochem.68.1.89

Kulaev I, Kulakovskaya T (2000) Polyphosphate and phosphate pump. Ann Rev Microbiol 54:709–734. doi:10.1146/annurev.micro.54.1.709

Kumble KD, Kornberg A (1996) Endopolyphosphatases for long chain inorganic polyphosphate in yeast and mammals. J Biol Chem 271(43):27146–27151

Lander N, Ulrich PN, Docampo R (2013) *Trypanosoma brucei* vacuolar transporter chaperone 4 (TbVtc4) is an acidocalcisome polyphosphate kinase required for in vivo infection. J Biol Chem 288(47):34205–34216. doi:10.1074/jbc.M113.518993

Lindner SN, Knebel S, Pallerla SR et al (2010) Cg2091 encodes a polyphosphate/ATP-dependent glucokinase of *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 87(2):703–713. doi:10.1007/s00253-010-2568-5

Lorenz B, Schroder HC (2001) Mammalian intestinal alkaline phosphatase acts as highly active exopolyphosphatase. Biochem Biophys Acta 1547(2):254–261

Mansurova SE, Shama AM, Sokolovskii V et al (1975) High-molecular polyphosphates in rat liver nuclei. Their behavior in the liver regeneration process. Dokl Akad Nauk SSSR 225(3): 717–720

Motta LS, Ramos IB, Gomes FM et al (2009) Proton-pyrophosphatase and polyphosphate in acidocalcisome-like vesicles from oocytes and eggs of *Periplaneta americana*. Insect Biochem Mol Biol 39(3):198–206. doi:10.1016/j.ibmb.2008.11.003

Nussenzveig RH, Oliveira PL, Masuda H (1992) Identification of yolk platelet-associated hydrolases in the oocytes of *Rhodnius prolixus*. Arch Insect Biochem Physiol 21(4):253–262. doi:10.1002/arch.940210402

Offenbacher S, Kline ES (1984) Evidence for polyphosphate in phosphorylated nonhistone nuclear proteins. Arch Biochem Biophys 231(1):114–123

Ogawa N, DeRisi J, Brown PO (2000) New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol Biol Cell 11(12):4309–4321

Oliveira DM, Gomes FM, Carvalho DB et al (2013) Yolk hydrolases in the eggs of *Anticarsia gemmatalis* hubner (Lepidoptera: Noctuidae): a role for inorganic polyphosphate towards yolk mobilization. J Insect Physiol 59(12):1242–1249. doi:10.1016/j.jinsphys.2013.09.008

Pavlov E, Aschar-Sobbi R, Campanella M et al (2010) Inorganic polyphosphate and energy metabolism in mammalian cells. J Biol Chem 285(13):9420–9428. doi:10.1074/jbc.M109.013011

Raikhel AS, Dhadialla TS (1992) Accumulation of yolk proteins in insect oocytes. Ann Rev Entomol 37:217–251. doi:10.1146/annurev.en.37.010192.001245

Ramos IB, Miranda K, de Souza W et al (2007) Calcium-regulated fusion of yolk granules is important for yolk degradation during early embryogenesis of *Rhodnius prolixus* Stahl. J Exp Biol 210(Pt 1):138–148. doi:10.1242/jeb.02652

Ramos IB, Miranda K, Pace DA et al (2010) Calcium- and polyphosphate-containing acidic granules of sea urchin eggs are similar to acidocalcisomes, but are not the targets for NAADP. Biochem J 429(3):485–495. doi:10.1042/BJ20091956

Ramos I, Gomes F, Koeller CM et al (2011) Acidocalcisomes as calcium- and polyphosphate-storage compartments during embryogenesis of the insect *Rhodnius prolixus* Stahl. PLoS One 6(11):e27276. doi:10.1371/journal.pone.0027276

Rao NN, Gomez-Garcia MR, Kornberg A (2009) Inorganic polyphosphate: essential for growth and survival. Ann Rev Biochem 78:605–647. doi:10.1146/annurev.biochem.77.083007.093039

Ruiz FA, Lea CR, Oldfield E et al (2004) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 279(43):44250–44257. doi:10.1074/jbc.M406261200

Secco D, Wang C, Shou H et al (2012) Phosphate homeostasis in the yeast *Saccharomyces cerevisiae*, the key role of the SPX domain-containing proteins. FEBS Lett 586(4):289–295. doi:10.1016/j.febslet.2012.01.036

Seidlmayer LK, Gomez-Garcia MR, Blatter LA et al (2012) Inorganic polyphosphate is a potent activator of the mitochondrial permeability transition pore in cardiac myocytes. J Gen Physiol 139(5):321–331. doi:10.1085/jgp.201210788

Shi X, Kornberg A (2005) Endopolyphosphatase in *Saccharomyces cerevisiae* undergoes post-translational activations to produce short-chain polyphosphates. FEBS Lett 579(9):2014–2018. doi:10.1016/j.febslet.2005.02.032

Shiba T, Itoh H, Kameda A et al (2005) Polyphosphate: AMP phosphotransferase as a polyphosphate-dependent nucleoside monophosphate kinase in *Acinetobacter johnsonii* 210A. J Bacteriol 187(5):1859–1865

Shirahama K, Yazaki Y, Sakano K et al (1996) Vacuolar function in the phosphate homeostasis of the yeast *Saccharomyces cerevisiae*. Plant Cell Physiol 37(8):1090–1093

Simbulan-Rosenthal CM, Gaur A, Sanabria VA et al (2015) Inorganic polyphosphates are important for cell survival and motility of human skin keratinocytes. Exp Dermatol. doi:10.1111/exd.12729

Snigirevskaya ES, Hays AR, Raikhel AS (1997) Secretory and internalization pathways of mosquito yolk protein precursors. Cell Tissue Res 290(1):129–142

Stotz SC, Scott LO, Drummond-Main C et al (2014) Inorganic polyphosphate regulates neuronal excitability through modulation of voltage-gated channels. Mol Brain 7:42. doi:10.1186/1756-6606-7-42

Talarico F, Brandmayr P, Giulianini P et al (2014) Effects of metal pollution on survival and physiological responses in *Carabus (Chaetocarabus) lefebvrei* (Coleoptera, Carabidae). Eur J Soil Biol 61:80–89

Tammenkoski M, Koivula K, Cusanelli E et al (2008) Human metastasis regulator protein H-prune is a short-chain exopolyphosphatase. Biochemistry 47(36):9707–9713. doi:10.1021/bi8010847

Travers RJ, Smith SA, Morrissey JH (2015) Polyphosphate, platelets, and coagulation. Int J Lab Hematol 37(Suppl 1):31–35. doi:10.1111/ijlh.12349

Tsutsumi K, Saito N, Kawazoe Y et al (2014) Morphogenetic study on the maturation of osteoblastic cell as induced by inorganic polyphosphate. PLoS One 9(2):e86834. doi:10.1371/journal.pone.0086834

Turbeck B (1974) A study of the concentrically laminated concretions, 'spherites' in the regenerative cells of the midgut of *Lepidopterous larvae*. Tissue Cell 6(4):627–640

Ulrich PN, Lander N, Kurup SP et al (2014) The acidocalcisome vacuolar transporter chaperone 4 catalyzes the synthesis of polyphosphate in insect-stages of *Trypanosoma brucei* and *T. cruzi*. J Eukaryot Microbiol 61(2):155–165. doi:10.1111/jeu.12093

Watabe S, Terada A, Ikeda T et al (1979) Polyphosphate anions increase the activity of bovine spleen cathepsin D. Biochem Biophys Res Commun 89(4):1161–1167

Watabe S, Ikeda T, Yago N (1996) Activation of cathepsin D by polyanionic compounds. Biochem Mol Biol Int 39(4):703–710

Wei ZZ, Vatcher G, Tin AH et al (2015) Positively-charged semi-tunnel is a structural and surface characteristic of polyphosphate-binding proteins: an in-silico study. PLoS One 10(4):e0123713. doi:10.1371/journal.pone.0123713

Weinstein BI, Li H (1976) Stimulation of chromatin template activity by the physiological macromolecule polyphosphate: a possible mechanism for eukaryotic gene derepression. Arch Biochem Biophys 175(1):114–120

Zakharian E, Thyagarajan B, French RJ et al (2009) Inorganic polyphosphate modulates TRPM8 channels. PLoS One 4(4):e5404. doi:10.1371/journal.pone.0005404

Zhang H, Ishige K, Kornberg A (2002) A polyphosphate kinase (PPK2) widely conserved in bacteria. Proc Natl Acad Sci U S A 99(26):16678–16683. doi:10.1073/pnas.262655199

Inorganic Polyphosphate and Its Chain-Length Dependency in Tissue Regeneration Including Bone Remodeling and Teeth Whitening

10

Toshikazu Shiba

Abstract

Inorganic polyphosphate (polyP) is a phosphate polymer that exists in mammalian cells and tissues. Recently, important physiological functions of polyP were discovered, and some functions were dependent on its molecular size (chain length). We found that medium-length polyP that is composed of 60 phosphate residues on an average has cell proliferation-enhancing activity in vitro, whereas no such activity is observed for short-chain polyP with average chain length of around 14 residues. The medium-chain polyP enhances fibroblast growth factor (FGF) function, accelerates tissue regeneration and bone formation, and inhibits bone resorption by osteoclasts. Long-chain polyP also shows similar activity as medium-chain polyP but is a more efficient inhibitor of bone resorption and iNOS expression. In contrast, short-chain polyP is an efficient stain control agent that removes tooth stains and prevents stain deposition on tooth surface. Pyrophosphate and tripolyphosphate, which are much shorter than short-chain polyP, have decreased stain removal and prevention of stain deposition efficiency. The efficiency of long- and medium-chain polyP molecules also decreases depending on the chain length. The maximum stain-removing activity was reported for ultraphosphate that has a highly cross-linked mesh-like structure including a branched PO_4 group in the molecule. These lines of evidence suggest

T. Shiba
Regenetiss Inc., 1-7-20, Higashi, Kunitachi, Tokyo 186-0002, Japan

Sleep & Circadian Neurobiology Laboratory, Stanford University School of Medicine, 3155 Porter Drive, Palo, Alto, CA 94304, USA

Division of Aesthetic Dentistry and Clinical Cariology, Department of Conservative Dentistry, Showa University, School of Dentistry,
2-1-1, Kitasenzoku, Ota-ku, Tokyo 145-6515, Japan
e-mail: shiba@regenetiss.com

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_10

that long- and medium-chain polyP are physiologically functional in mammals, and short-chain polyP and ultraphosphate are effective as stain-controlling agents for human teeth.

10.1 Preparation of Highly Polymerized PolyP

Inorganic polyphosphates (polyP) are condensed phosphates that are generated by polymerizing three or more PO_4 tetrahedra to share an oxygen atom included in other PO_4 tetrahedra (Averbuch-Pouchot and Durif 1996). The condensed phosphates are classified into polyP, metaphosphates, and ultraphosphates based on the molar ratio of M_2O/P_2O_5 ("M" represents a monovalent metal typified by an alkali metal). PolyP has an M_2O/P_2O_5 molar ratio (R) that satisfies the condition, $2 > R > 1$, and is a linear compound represented by Formula (10.1):

$$M_{m+2}P_mO_{3m+1} \tag{10.1}$$

("m" represents an integer of 2 or greater.)

The metaphosphate has an M_2O/P_2O_5 molar ratio $R = 1$ and is a cyclic or extremely long linear compound represented by Formula (10.2):

$$\left(MPO_3\right)_n \tag{10.2}$$

("n" represents an integer of 3 or greater.)

The abovementioned polyP and metaphosphate are together referred to as polyP.

Since polyP is a phosphate polymer, its molecular weight depends on the length of the phosphate residue chain. Commercially available polyP that is used as a food additive and cosmetic ingredient is commonly referred to as metaphosphate, hexametaphosphate, or tripolyphosphate. However, the chain lengths of these polyP species are shorter than the polyP molecule naturally found in living organisms. Generally, the average chain length of polyP found in mammalian cells and tissues is around 60–100 phosphate residues (Müller et al. 2009; Ruiz et al. 2004; Moreno-Sanchez et al. 2012), whereas the average chain length of commercially available hexametaphosphate and metaphosphate is less than 10 phosphate residues. Pyrophosphate and tripolyphosphate that are composed of only two and three phosphate residues, respectively, are also used as food additives; however, they do not have remarkable physiological function.

To investigate polyP functions in mammalian cells, longer-chain polyP polymerizing more than 60 phosphate residues on average was purified from food additive-grade metaphosphates. Food additive-grade metaphosphates contain a little amount of longer-chain polyP although the percentage of the longer chain is dependent on manufacturer. However, normally a small percentage of polyP with more than 60 phosphate residues can be extracted from metaphosphates. Even the food additive-grade hexametaphosphates are not pure hexamers (6 mer) and contain polyP with both more than and less than six phosphate residues, since the synthesis and purification of molecules that can polymerize exactly six phosphate residues are difficult.

Therefore, hexametaphosphate is a mixture of tri-, tetra-, penta-, hexa-, hepta-, octa-, and more highly polymerized phosphate polymers.

An easy method to purify long-chain polyP from metaphosphate is described below (Shiba et al. 2004). Metaphosphate solution (10 w/v%) was prepared and ethanol was added to the final concentration of 14 w/v%. The solution was mixed well and kept for more than 30 min at room temperature. To separate the ethanol phase (upper layer) and polyP solution phase (lower layer), centrifugation was performed at 10,000 x *g* for 20 min. The resultant polyP solution phase (lower layer) was collected and washed by mixing with equal volume of 70% ethanol. After centrifugation (10,000×g for 20 min at room temperature), polyP solution phase (lower layer) was collected. The resultant polyP solution has high viscosity and normally contains more than 50% polyP. Long-chain polyP could be purified by repeating this ethanol extraction and washing process. Using this size fractionation protocol, we prepared long-chain polyP with an average chain length of 130 phosphate residues and medium-chain polyP with an average chain length of 60 phosphate residues. Short-chain polyP with an average chain length of 14 phosphate residues are also obtained through this fractionation protocol (Fig. 10.1).

Recently, we succeeded in synthesizing highly polymerized polyP molecules with 60–300 phosphate residues in industrial scale. For synthesis of long-chain

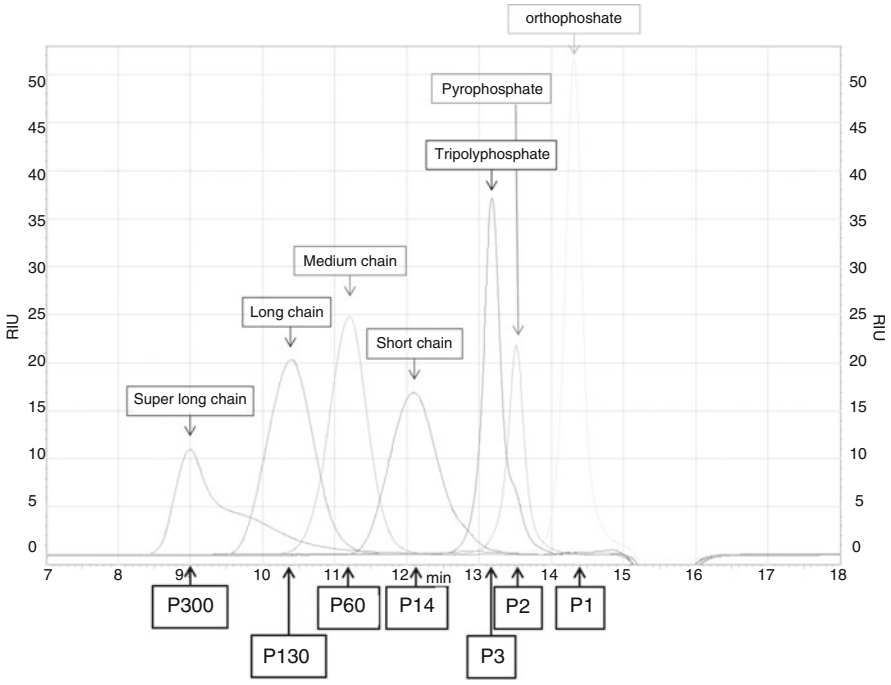

Fig. 10.1 HPLC gel filtration analysis of polyP. P1, P2, P3, P14, P60, P130, and P300 represent the number of average phosphate residues polymerized

polyP at the industrial scale, sodium or potassium phosphate is rapidly heated at high temperature (more than 600 °C), and the melted phosphate solution is rapidly cooled at room temperature. Depending on the heating temperature and time, polyP of various chain lengths could be synthesized. Combining of this heating method and subsequent ethanol extraction, we succeeded in obtaining superlong-chain polyP with ~300 phosphate residues on an average (Fig. 10.1). We believe that this long-chain polyP may be useful for developing new medical and healthcare products.

10.2 Physiological Functions of Long-, Medium-, and Short-Chain PolyP

Using the long-, medium-, and short-chain polyP that were prepared by size fractionation as described above (Fig. 10.1), we performed functional analyses of each polyP group. The physiological and biological functions of long-, medium-, and short-chain polyP that were discussed in Sect. 10.10 are summarized in Fig. 10.2.

The long-chain polyP has antifungal activity. In addition, inducible nitric oxide synthase (iNOS) expression in macrophages and bone resorption by osteoclasts are both suppressed by the long-chain polyP (Harada et al. 2013a, b). The medium-chain polyP has almost the same chain length as its counterpart in mammalian cells and tissues and has FGF-stabilizing functions, including enhancement of cell proliferation (Shiba et al. 2003; Kawazoe et al. 2008), bone regeneration (Kawazoe et al. 2004;

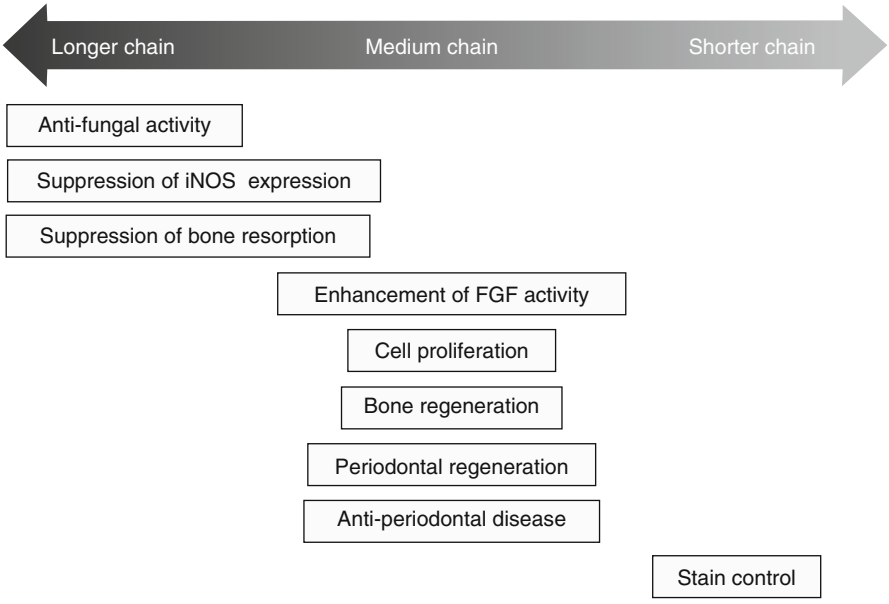

Fig. 10.2 A summary of chain length-dependent polyP functions

Hacchou et al. 2007; Tsutsumi et al. 2014), and periodontal tissue regeneration (Shiba et al. 2004; Hacchou et al. 2007; Yamaoka et al. 2008). In addition, medium-chain polyP has a therapeutic benefit in periodontal disease (Yamaoka et al. 2008). The short-chain polyP is an effective stain removal and prevention of stain deposition on teeth surface.

Other biological functions in long- and medium-chain polyP are also reported. For example, medium-chain polyP in platelets plays an important role in blood coagulation (Ruiz et al. 2004), by supporting protein folding like chaperon molecules (Gray et al. 2014). Segawa et al. demonstrated that the enhancement of epithelial barrier function and maintenance of intestinal homeostasis resulted from probiotic-derived long-chain polyP (Segawa et al. 2011). They isolated polyP as the cytoprotective compound from a *Lactobacillus* strain.

As described above, polyP has different functions depending on its chain length. Especially, long- and medium-chain polyP have important physiological functions in mammals, whereas short-chain polyP does not have any remarkable physiological function but is useful for stain control. These functions of polyP and their chain-length dependency will be discussed in the following pages.

10.3 Enhancement of Tissue Regeneration by Medium- and Long-Chain PolyP

We previously reported that tissue regeneration induced by medium- and long-chain polyP is based on the stabilization of FGF-1 and FGF-2 and enhancement of binding affinity between FGF and its cell-surface receptor (Shiba et al. 2003). FGF-1 and FGF-2 are well-known cytokines that accelerate tissue regeneration and have already been used for acceleration wound healing including treatment of burn scars and pressure ulcers (Akita et al. 2006). Recently, FGF-2 was also applied in periodontal disease to regenerate alveolar bone (Murakami 2007). However, FGF-2 itself is an unstable protein and endogenous FGF-2 is degraded rapidly within tissues.

Both medium- and long-chain polyP are effective to prolong biological half-life of FGF-2 and preventing its degradation (Shiba et al. 2003). The medium- and long-chain polyP enhance cell proliferation, whereas almost no enhancement of proliferation was observed with short-chain polyP compounds (Fig. 10.3). Thus, medium- and long-chain polyP could enhance FGF activity by strengthening the binding affinity between FGF and its receptor. Since the efficiency of enhancing cell proliferation is slightly better with medium-chain polyP than with long-chain polyP (Fig. 10.3), we used medium-chain polyP to evaluate polyP function on tissue regeneration. In any case, the enhancement of fibroblast proliferation, stabilization of FGF, and functional augmentation of FGF (enhancement of binding affinity between FGF and its receptor) are dependent on the chain length of polyP. Since spontaneous degradation of long-chain polyP could generate medium-chain molecules of polyP, these medium-chain degradation products derived from long-chain polyP may enhance cell proliferation.

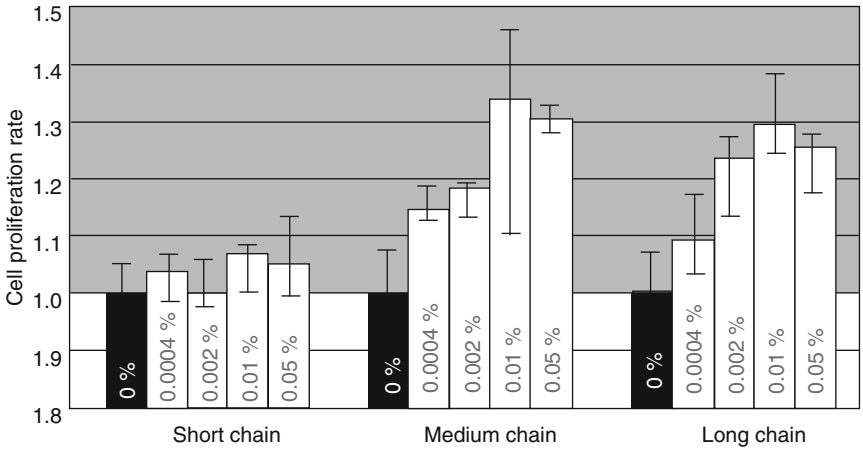

Fig. 10.3 Chain length-dependent enhancement of cell proliferation by polyP. Cell proliferation rate was calculated in terms of a relative value when the level of untreated cells (0 %) was defined as 1. Percentage values represent polyP concentration added to the culture media. Cell culture and basic proliferation assay were performed as described by Shiba et al. (2003)

10.4 Acceleration of Cell Calcification by Medium-Chain PolyP

As an in vitro study, the effect of medium-chain polyP was investigated on MC3T3-E1 cells (Kawazoe et al. 2004). MC3T3-E1 is an osteoblastic cell line isolated from the mouse skull and is commonly used in bone differentiation experiments. MC3T3-E1 cells were cultured for 35 days with medium-chain polyP, and mRNA expression of bone differentiation marker genes such as osteopontin and osteocalcin was monitored. Ten days after polyP treatment, the expression osteopontin mRNA was 3.5–10 times higher than that of untreated cells. Osteocalcin expression in polyP-treated cells was also elevated by two- to threefold compared to untreated cells. Alkaline phosphatase activity increased by 1.5- to 2-fold by 10–21 days after polyP treatment, but the level of enhancement was not higher than that induced by the positive control (10 mM β-glycerophosphate and 50 μg/ml ascorbic acid). Furthermore, cell calcification was also observed after treatment of 31 days, by alizarin red staining. These findings support that bone differentiation of MC3T3-E1 cells was induced by medium-chain polyP.

To investigate the effect of medium-chain polyP on undifferentiated cells, human mesenchymal stem cells (MSC) and normal human dental pulp cells (HDP) were employed (Kawazoe et al. 2008). Similar to normal human fibroblasts, cell proliferation of both MSC and HDP was enhanced by treatment with polyP. Interestingly, FGF-2 was detected on the cell surface after 40 h of incubation with medium-chain polyP, whereas no FGF-2 was observed in untreated cells. In addition, phosphorylation of ERK1/2, a MAP kinase, was accelerated in cells treated with medium-chain polyP, reaching 1.7–2.2-folds compared to untreated cells after 48 h of treatments.

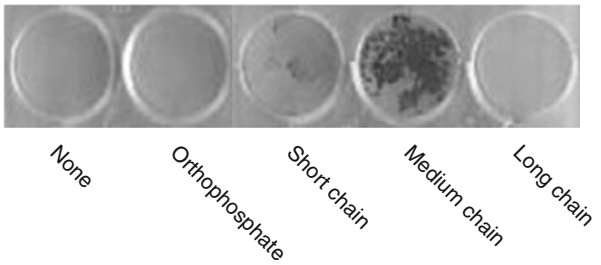

Fig. 10.4 Calcification of MSCs by polyP. Cells were cultured with or without 1 mM polyP of various chain lengths or 1 mM-phosphate buffer (orthophosphate) for 24 days and then stained with alizarin red as described by Kawazoe et al. (2008)

These results indicate that medium-chain polyP enhances proliferation of MSC and HDP by activating the FGF signaling pathway.

Similar to the results obtained with MC3T3-E1 cells, mRNA expression of osteopontin and osteocalcin was induced by medium-chain length polyP in MSC and HDP after 20 h of incubation. Microarray analysis revealed that mRNA expression of osteoprotegerin, which is an inhibitory factor of osteoclast differentiation, was also elevated by more than threefold after medium-chain polyP treatment. Cell calcification was also observed by alizarin red staining 17 days after medium-chain polyP treatment. Since cell calcification was not observed when the cells were treated with short- or long-chain polyP, the induction of bone differentiation depends on the chain length of polyP (Fig. 10.4). All these phenomena strongly support that bone differentiation of undifferentiated stem cells like MSC and HDP was induced by medium-chain polyP treatment in vitro.

10.5 Bone Regeneration by Medium-Chain PolyP-Adsorbed Hydroxyapatite In Vivo

Yuan et al. (2009) and Morita et al. (2010) demonstrated that medium-chain polyP-adsorbed interconnected porous calcium hydroxyapatite (IP-CHA) accelerated bone regeneration in vivo. Various concentrations of medium-chain polyP were adsorbed to IP-CHA by simply soaking IP-CHA into 1–50 % solutions of medium-chain polyP. The polyP-adsorbed IP-CHA was then dried under vacuum for 2 h and further dried at 37 °C. Approximately 2.4–112 μg of medium-chain polyP was adsorbed onto 1 mg of IP-CHA. These medium-chain polyP adsorbed onto IP-CHA were implanted into bone sockets in the femurs (diameter, 3 mm × depth, 5 mm) of New Zealand white rabbits. Two weeks after implantation, the femurs were harvested and histologically analyzed. Newly formed lamellar bone was found in the pores in all groups, but it was quite obvious that much more bone was formed in the 25 and 50 % medium-chain polyP-adsorbed IP-CHA (Fig. 10.5). Based on these results, the bone regeneration ratio was calculated as the ratio of newly formed bone to that of total regenerated tissue. The bone regeneration ratio was 53.5 and 58.6 %

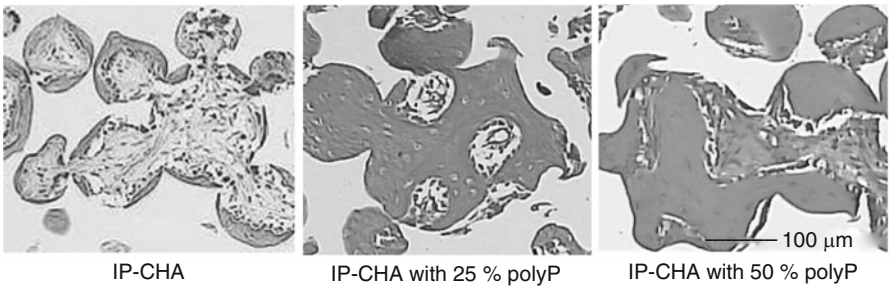

Fig. 10.5 Acceleration of bone formation by polyP (Morita et al. 2010). Histological specimens at 2 weeks after implantation were stained with hematoxylin and eosin. Newly formed lamellar bone was found at the marginal area, and higher new bone formation was clearly observed in the IP-CHA with both 25 and 50 % polyP groups. Scale bar represents 100 μm

in the 25 and 50 % medium polyP-adsorbed IP-CHA, respectively. These values were significantly higher ($p < 0.05$) than the bone regeneration ratio of IP-CHA without polyP (36.1 %).

The acceleration of bone regeneration by medium-chain polyP could be a result of FGF2 stabilization and enhancement of affinity between FGF and its receptor. Medium-chain polyP also accelerated osteoblast differentiation and calcification of cells going into hydroxyapatite through the interconnected pores. Since 25 and 50 % polyP-adsorbed IP-CHA significantly enhance bone regeneration, higher-concentration medium-chain polyP seems to be effective on the regeneration. Especially, 50 % polyP-adsorbed IP-CHA contains 112 μg of polyP in 1 mg of IP-CHA, suggesting that concentration of medium-chain polyP adsorbed in IP-CHA may correspond to more than several percent of the total weight of IP-CHA. The local concentration may also correspond to several hundred molars in terms of phosphate residues. These results also suggest that high concentration of medium-chain polyP is not toxic to cells including osteoblasts in vivo since no ominous findings were observed in this animal experiment.

10.6 Regeneration of Alveolar Bone by Medium-Chain PolyP

To examine the effect of medium-chain polyP on periodontal regeneration in vivo, animal experiments were performed using Wistar rats (Shiba et al. 2004; Hacchou et al. 2007). The mucoperiosteal flaps on both sides of the mandible and buccal osseous dehiscences were created by removal of approximately 3 × 3 mm of the bone covering the roots of the bilateral mandibular first molars. A reference notch was marked in the root surface at the levels of surgically reduced bone crest by means of a round burr. The buccal mucoperiosteal flaps were readapted. A carboxy-methyl cellulose (CMC) solution containing 1 w/v% of medium-chain polyP (polyP-CMC solution) was applied to the right sulcus 5 days a week after surgery. On the left side, CMC alone was applied as control. Rats were sacrificed at 1, 2, or 3 weeks following surgery. The mandibles were dissected, fixed in formalin, and

decalcified in EDTA for 1 month. Serial sections were cut with a cryostat in a buccolingual direction and then stained with hematoxylin and eosin.

Areas of newly formed bone at 2 weeks after surgery had regenerated over the reference notch marked on the root surface. The mean areas of observed alveolar bone repair in the medium-chain polyP-treated group and control group were 3.2 ± 0.6 mm^2 and 0.9 ± 0.2 mm^2, respectively. Thus, the new bone formation in the medium-chain polyP-treated group was 3.6-fold higher than in the control group ($P < 0.01$). These results suggest that medium-chain polyP facilitates alveolar bone regeneration in rats.

To confirm the effect of medium-chain polyP on periodontal regeneration, we designed an alveolar bone defect model using the beagle dog in a manner similar to the rat model described above. After the surgery, periodontal pockets of the bone defected area were washed everyday with phosphate-buffered saline containing 1 % medium-chain polyP for 2 weeks (polyP-treated group, $n = 5$). In the control group, the pockets were washed with phosphate-buffered saline alone ($n = 5$). Histological samples were stained with hematoxylin and eosin, and the areas of regenerated alveolar bone and cementum were measured. Histological analysis revealed that the regenerated alveolar bone area of medium-chain polyP-treated group was 2.54 ± 1.90 mm^2, whereas that of the control group was 1.08 ± 0.88 mm^2. This suggests that medium-chain polyP accelerates alveolar bone regeneration. In addition, the ratio of alveolar bone area between the nonsurgical region and the regenerated region after surgical treatment (the area of alveolar bone of surgical region was divided by the area of alveolar bone of no surgical region) was 28 ± 2 % in polyP-treated group. In contrast, the ratio of alveolar bone area was 17 ± 0.9 % in the control group. These results also suggest that alveolar bone regeneration was enhanced by medium-chain polyP.

In addition to alveolar bone regeneration, significant new cementum formation was also observed in the medium-chain polyP-treated group (Fig. 10.6). The area of new cementum formed in the medium-chain polyP-treated group was 0.82 ± 0.54 mm^2, whereas no cementum formation was observed in the control group. Since cementum formation would occur before alveolar bone formation, washing the periodontal pockets with medium-chain polyP could be an effective strategy to induce alveolar bone regeneration.

10.7 Clinical Test for Periodontal Diseases

Using polyP-CMC solution, a clinical trial was performed in periodontal disease patients (Yamaoka et al. 2008). Patients older than 27 years of age in general good health, with previously untreated periodontitis and an incisor or premolar probing depth ≥4 mm with bleeding on probing (BOP), who volunteered to take medium-chain polyP for experimental therapy were selected. Patients were randomly allocated to the treatment groups by an automated assignment procedure using numbered containers concealed from the investigator. Neither the patient nor the operator knew whether the patient received polyP-CMC or placebo as control (CMC alone).

The medium-chain polyP-treated group comprised of 16 patients who washed their periodontal pockets with the polyP-CMC solution everyday. On the other hand, the 17

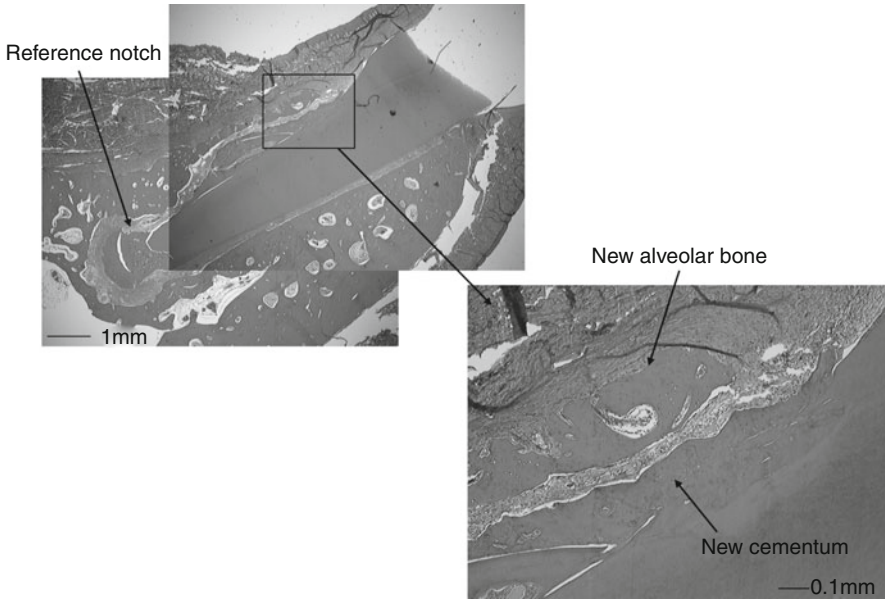

Fig. 10.6 Alveolar bone regeneration and new cementum formation by polyP in beagle dogs. Two weeks of treatment with medium-chain polyP accelerates new cementum formation and alveolar bone regeneration

patients in the control group washed their periodontal pockets with CMC solution alone. As shown in Fig. 10.7, the patients themselves washed their periodontal pockets once a day, and after a week, the probing pocket depth (PPD) and BOP were evaluated. In the medium-chain polyP-treated group, five patients showed improvement in both PPD and BOP, whereas in the control group, only one patient showed improvement. This suggests that medium-chain polyP could be effective for treatment of periodontal diseases. Furthermore, the average age of the patients with simultaneous improvement of gingival index (degree of inflammation in gingival area) and BOP in the medium-chain polyP-treated group was higher than that of patients who showed the improvements in the control group, suggesting that medium-chain polyP is more effective in older periodontal disease patients (over the age 51 years). Although one case in the polyP-CMC group showed possible bone regeneration at the 13th week, significant bone regeneration was not observed in other patients in the polyP-CMC group.

10.8 Inhibition of Osteoclastic Bone Resorption by Long-Chain PolyP

As described above, many data support the acceleration of bone regeneration by medium-chain polyP. In addition, we recently found that long-chain polyP inhibits the bone resorption activity of osteoclasts (Harada et al. 2013a). The effects of polyP with various chain lengths on bone resorption were evaluated as follows.

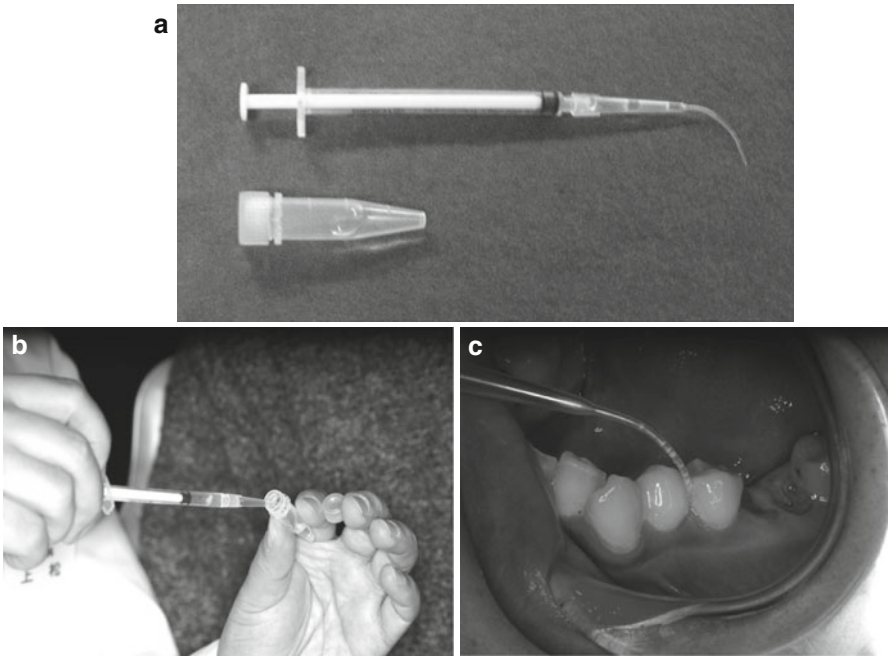

Fig. 10.7 Clinical trial of polyP for periodontal disease. Panel (**a**): PolyP-CMC solution and applicator. Panel (**b**): Taking polyP-CMC solution in the applicator just before washing the periodontal pockets. Panel (**c**): Washing the periodontal pockets using the applicator

Osteoclast precursor cells prepared from rat bone marrow were cultured with macrophage colony-stimulating factor (M-GSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) to induce differentiation from precursor cells into osteoclasts on calcium phosphate-coated plates. Cells were incubated for 3 days and further incubated for 4 days with or without polyP of various chain lengths (1 mM) in calcium phosphate-coated plates. Bright-field microscopic images of five randomly selected fields per plates were obtained, and the pit area (area of bone resorbed by osteoclasts) was measured. In addition, to identify osteoclasts, cells cultured with M-GSF and RANKL for 5 days were stained for tartrate-resistant acid phosphatase (TRAP) activity.

When 1 mM polyP was added on day 3 of the culture, the number of resorption pits present was remarkably decreased by day 7. In particular, superlong-chain polyP (average chain length of ~300 phosphate residues) almost completely inhibited pit formation. Hacchou et al. (2007) also analyzed the effect of polyP on bone resorption using co-cultures of mouse calvarial osteoclasts and tibial bone marrow macrophages (Yang et al. 2005) and observed an apparent decrease in the number of resorption pits after treatment with long-chain polyP (Fig. 10.8). These results suggest that long-chain polyP is a strong inhibitor of bone resorption activity compared to short-chain polyP. Since the number of TRAP-positive multinuclear cells or osteoclasts did not decrease after the addition of polyP into

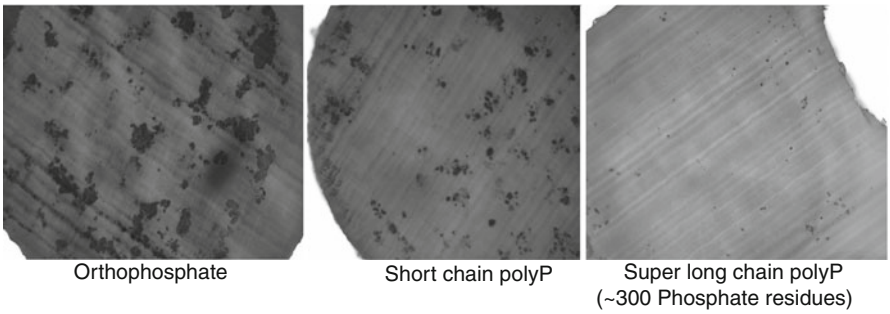

Fig. 10.8 Suppression of bone resorption by long-chain polyP (Hacchou et al. 2007). Bone-absorbed areas formed by osteoclasts were visualized as darker dots (pits)

the culture media, polyP could not inhibit osteoclast differentiation. In contrast, the long-chain polyP significantly increased the number of TRAP-positive multinuclear cells.

Interestingly, TRAP activity is strongly inhibited by long-chain polyP. This inhibition is dependent on the polyP chain length, and the IC_{50} of polyP against original TRAP activity was 0.66 μM with an average chain length of more than 750 phosphate residues, whereas the IC_{50} of polyP with shorter average chain length of 15 phosphate residues was 846 μM (Harada at al. 2013a). The chain-length dependency of TRAP inhibition is consistent with the results that long-chain polyP is a potent inhibitor of bone resorption by osteoclasts.

TRAP is abundantly expressed on osteoclasts and plays an important role in osteoclastic bone resorption. For example, the resorbed bone matrix, such as type I collagen, is endocytosed into osteoclasts and is likely to be further degraded by reactive oxygen species derived from TRAP (Halleen et al. 1999). In addition, mice deficient in *acp5*, which is the TRAP-encoding gene, were reported to show mild osteopetrosis (Hayman et al. 1996), and treatment of osteoclasts with TRAP inhibitors reduced bone resorption (Zaidi et al. 1989). Thus, the inhibition of bone resorption by long-chain polyP could be a result of inhibition of TRAP activity. These lines of evidence indicate that polyP is not only an accelerator for bone formation but also an inhibitor of bone resorption.

10.9 A Probable Model of PolyP Function on Bone Regeneration

Bone remodeling is regulated by the coordinated action between osteoblasts and osteoclasts. Recently, Omelon et al. reported that osteoclasts synthesize and secrete polyP (Omelon et al. 2009). Taking into account the effect of polyP on osteoblasts and osteoclasts, a probable model for polyP function on bone remodeling is as follows (Harada et al. 2013a): long-chain polyP synthesized and secreted by osteoclasts inhibits bone resorption activity of osteoclasts themselves; shortly thereafter,

long-chain polyP is degraded and medium-chain polyP is formed. This medium-chain polyP induces bone formation by osteoblasts. Finally, the medium-chain polyP is also degraded and might be utilized as a phosphate source in hydroxyapatite formation for new bone. Although further analyses are necessary to elucidate the details of polyP function on bone remodeling, polyP may have a regulatory function in the coordinated action between osteoblasts and osteoclasts depending on its chain length.

10.10 Other Biological Functions of Long-Chain PolyP

In response to infection, macrophages produce a series of inflammatory mediators, including nitric oxide (NO), to eliminate pathogens. Harada et al. (2013b) reported that long-chain polyP suppresses iNOS expression induced by lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, in mouse peritoneal macrophages. PolyP with longer chains is more potent than that with shorter chains in suppressing LPS-induced iNOS expression at the mRNA level. In addition, polyP decreased LPS-induced NO release. In contrast, polyP did not affect LPS-induced release of TNF that is another inflammatory mediator. Although the detailed mechanism remains to be elucidated, long-chain polyP somehow regulates innate immunity. Since medium- and long-chain polyP composed of 60–100 phosphate residues accumulate in the granules of platelets and mast cells (Müller et al. 2009; Moreno-Sanchez et al. 2012; Ruiz et al. 2004), these cells secrete polyP into the extracellular space, which may serve as an intercellular signaling molecule in innate immunity.

Long-chain polyP also has antifungal activity. It inhibits the growth of *Candida albicans*, *Saccharomyces cerevisiae*, and *Trichophyton mentagrophytes* (Shiba et al. unpublished results). To examine the antifungal activity of polyP, we prepared potato dextrose agar plates containing polyP of various chain lengths at various concentrations and placed a disk of *T. mentagrophytes* fungal filaments at the center of the agar plates. As shown in Fig. 10.9, long-chain polyP inhibits the growth of *T. mentagrophytes*. Especially, polyP with more than 60 phosphate residues on an average is a potent inhibitor of fungal growth. Thus, medium- and long-chain polyP could be useful to prevent conditions like athlete's foot.

10.11 Effect of Short-Chain PolyP on Stain Control of Human Teeth; Evaluation Using Hydroxyapatite as Human Teeth Model

While long- and medium-chain polyP have various biological functions, short-chain polyP has a special function in removal of stains (coloration on the teeth) from the teeth. Short-chain polyP is also effective at prevention of stain deposition on the teeth. Interestingly, the effect of short-chain polyP on both stain removal and prevention of stain deposition is strongly dependent on its chain length.

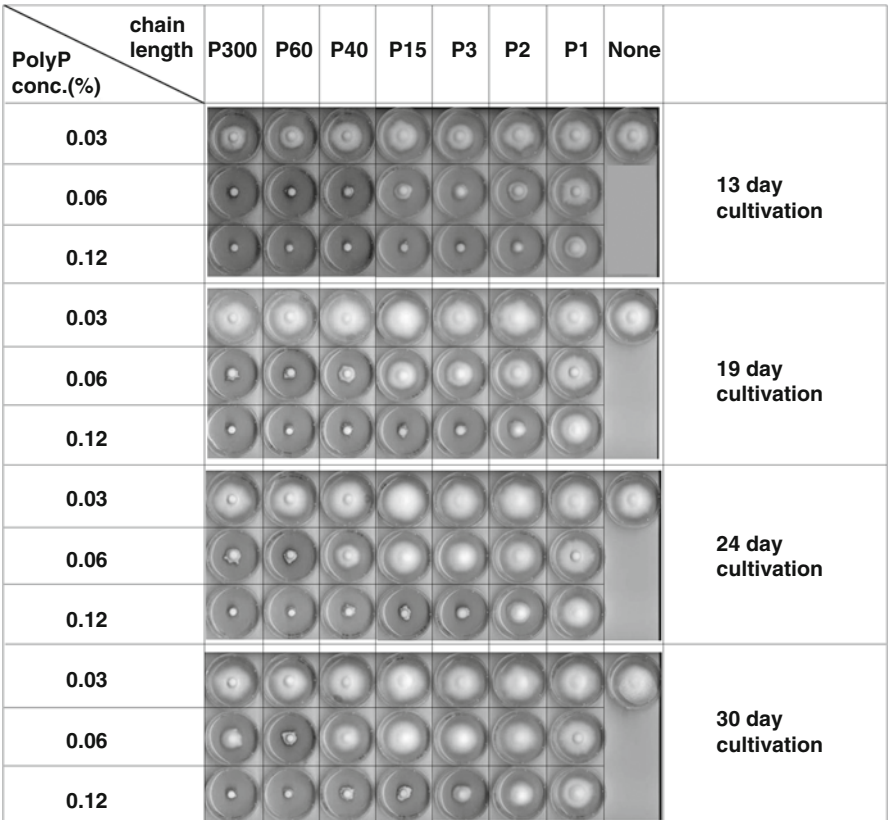

Fig. 10.9 Growth inhibition of *T. mentagrophytes* by long-chain polyP. P1, P2, P3, P15, P40, P60, P130, and P300 represent the average chain length (number of phosphate residues polymerized)

To evaluate the effect of polyP of various chain lengths on stain removal and prevention of stain deposition, hydroxyapatite powder was used as an artificial tooth model. Original protocols for stain removal and prevention of stain deposition test were described previously (Baig et al. 2002). Hydroxyapatite powder (150 mg) was put into a centrifuge tube, and 10 ml of purified water was added. The centrifuge tube was tipped over for washing the hydroxyapatite powder with water. This solution was separated by centrifugation (1,500 × g for 2 min) to remove the supernatant. To the hydroxyapatite pellet obtained after the supernatant was removed, 5 ml of tea extract was added, and the centrifuge tube was tipped over, and hydroxyapatite was mixed with the tea extract. Thereafter, the resulting solution was separated by centrifugation (1,500 × g for 2 min), and the supernatant was again removed.

In order to wash off the tea stain, which was not adsorbed to hydroxyapatite, 10 ml of purified water was added to the hydroxyapatite pellet, and the centrifuge tube was tipped over for washing the hydroxyapatite with water for 1 min. After the supernatant was separated by centrifugation (1,500 x *g* for 2 min), polyP solutions

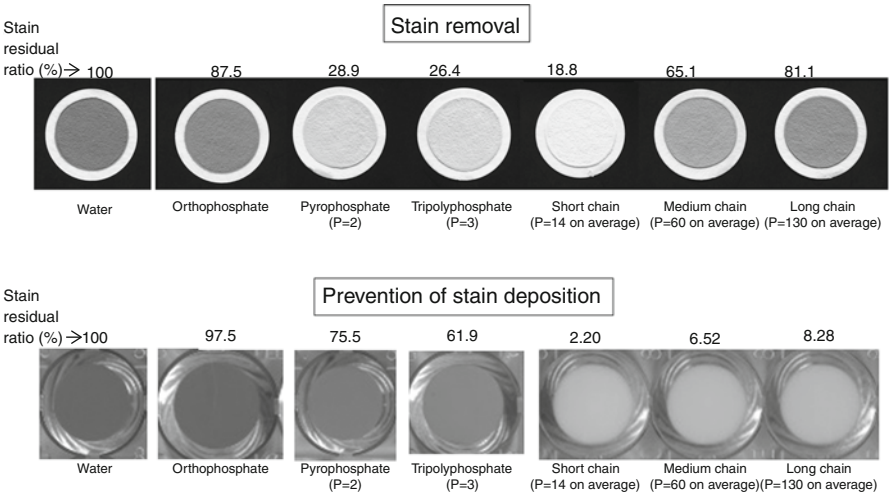

Fig. 10.10 Efficiency of stain removal and prevention of stain deposition by polyP of various chain lengths. Stain residual ratio was calculated in terms of a relative value as described in the legend of Table 10.1

at various chain lengths were added to the hydroxyapatite pellet. The centrifuge tube was then tipped over for 1 min to mix them, and the hydroxyapatite was separated from the polyP solution by centrifugation (1,500× g for 2 min). After the supernatant was removed, 10 ml of purified water was added to the hydroxyapatite pellet, and the pellet was washed. After this washing procedure was repeated again, hydroxyapatite was subjected to suction filtration. The filtered hydroxyapatite powder was air-dried and scanned with an image scanner. The image was then inverted to negative, and the color density was calculated by an image analysis program.

The results are shown in Fig. 10.10. The most effective molecule for stain removal was the short-chain polyP with a chain length of 14 phosphate residues on an average. Almost no stain removal activity was detected in orthophosphate (monomer) and in long-chain polyP with an average chain length of 130 phosphate residues. Although pyrophosphate (dimer) and tripolyphosphate (trimer) had weak stain removal activity, their efficiency was lower than that of short-chain polyP. These results clearly indicate that the optimum range of chain length for stain removal efficiency was very narrow.

We also evaluated the effect of polyP of various chain lengths on the prevention of stain deposition. For testing the prevention of stain deposition, 30 mg of hydroxyapatite powder was put into each 2 ml centrifuge tube, and 1 ml of each test solution containing various chain-length polyP or purified water (negative control) was added into the tubes. The tube was inverted to mix and then centrifuged at 1,500×g for 2 min to remove the supernatant. The resultant hydroxyapatite pellets were washed with 2 ml of purified water, and the supernatant was removed. This washing procedure was repeated twice in total; then 1 ml of tea extract was added to each tube and the tube was inverted for 1 min. After the supernatant was removed, each

hydroxyapatite pellet was washed with 2 ml of purified water. Finally, 2 ml of purified water was added to each hydroxyapatite pellet and inverted for 1 min for a hydroxyapatite suspension. Then each suspension was transferred into 96-multi-well plate, and the color of each hydroxyapatite powder was scanned from the bottom of the multi-well plate using an image scanner. The ratio of the prevention of stain deposition was calculated from the scan data.

The efficiency of prevention of stain deposition is shown in Fig. 10.10. Similar to the stain removal activity, short-chain polyP with the chain length of 14 phosphate residues on an average was better than the other size for stain prevention. Orthophosphate, pyrophosphate, and tripolyphosphate were not superior to short-chain polyP in preventing stain deposition. Although the stain prevention efficiency of short-chain polyP is slightly better than that of medium- and long-chain polyP, the difference in their efficiency was not greater than the difference in stain removal efficiency. These results suggested that short-, medium-, and long-chain polyP have almost the same level of efficacy at prevention of stain deposition.

10.12 Evaluation of Stain Removal and Prevention of Stain Deposition Activity Using Detached Human Teeth

To confirm the stain removal activity of short-chain polyP on natural tooth surface, detached human teeth were immersed in concentrated tea extract solution for more than 24 h, and colored teeth were washed with water to remove the excess tea solution. CMC gel containing short-chain polyP (1 w/w%) was then applied to the stained tooth for 30 min at 37 °C. As a control, CMC gel without polyP was also applied on the stained tooth. After the teeth were washed with water, the stain removal efficiency in the short-chain polyP-treated tooth and control tooth was evaluated. As shown in Fig. 10.11, the tooth stain was almost removed by short-chain polyP, whereas almost no stain was removed in the control.

Using the similar procedure, we confirmed the efficiency of prevention of stain deposition by the short-chain polyP. First, detached human teeth were treated with 32 % hydrogen peroxide solution for 24 h and completely bleached. To completely remove hydrogen peroxide, the teeth were washed with water. Then the CMC gel with or without short-chain polyP was put on the teeth surface for 15 min at 37 °C. After the teeth were washed with water, the teeth were immersed in concentrated tea extract solution for 1 h. After the teeth were washed again with water, they were immersed in tea solution again for 1 h. The stain deposited on the short-chain polyP-treated tooth and that on the control tooth was evaluated. As shown in Fig. 10.11, tooth stain was not deposited on short-chain polyP-treated tooth even after 2 h of exposure to the tea extract solution.

The possible mechanism for stain removal and prevention of stain deposition is based on the affinity between polyP and the tooth surface composed of hydroxyapatite. Short-chain polyP could have the highest affinity with hydroxyapatite. The affinity between short-chain polyP and hydroxyapatite may be dependent on the negative charge of polyP molecule. However, the affinity is not dependent on the

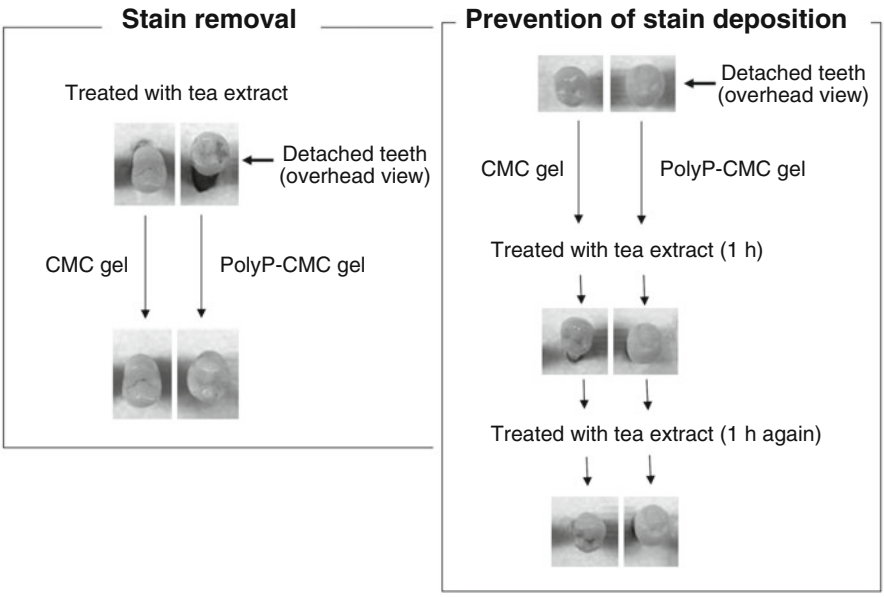

Fig. 10.11 Evaluation of stain control activity by short-chain polyP on detached human teeth

strength of the negative charge alone, since the long-chain polyP whose negative charge is higher than that of the short chain showed weaker activity in stain removal and prevention of stain deposition than that of short-chain polyP. Short-chain polyP may bind with hydroxyapatite not only by electric charge but also by any other mode of binding which may depend on polymer size.

10.13 Mesh-Type PolyP, Ultraphosphate, Is the Most Powerful Stain Remover

Ultraphosphate is a kind of polyP that has a highly cross-linked mesh-like structure including a branched PO_4 group in the molecule and is also known as a strong chelating agent (Averbuch-Pouchot and Durif 1996). While polyP including metaphosphates have linear or branched structures, ultraphosphate is highly branched and the M_2O/PO_4 molar ratio (R) satisfies $1 > R > 0$ ("M" represents a monovalent metal typified by an alkali metal). It has conventionally been used as a food additive for food preservation and as an inhibitor of food color change (Koyasu et al. 2014).

To compare the efficiency of stain control between sodium ultraphosphate and short-chain polyP, the stain removal and prevention of stain deposition activity were evaluated using hydroxyapatite powder (Shiba et al. 2015). The method for this comparison using hydroxyapatite was the same as described in Sect. 10.11 (Koyasu et al. 2014; Shiba et al. 2015). The stain removal efficiency of sodium ultraphosphate is slightly higher than that of short-chain polyP (Table 10.1). To maximize

Table 10.1 Comparison of stain removal efficiency among short-chain polyP, ultraphosphate, and size-fractionated ultraphosphates

	Average chain length (number of phosphate residues)	Stain residual ratio[a]%
Short-chain polyP	14.0	18.86
Ultraphosphate	17.9	10.18
Size-fractionated ultraphosphate	18.2	14.77
	11.8	12.57
	11.3	7.74
	10.2	5.03
	8.7	7.63
	7.6	12.62

[a]Stain residual ratio was calculated in terms of a relative value when the color density of colorized hydroxyapatite washed by water was defined as 100 % and the color density of untreated hydroxyapatite was defined as 0 %

stain removal activity of ultraphosphate, we prepared size-fractionated ultraphosphate and found that the optimal chain length for stain removal is around 10.2 phosphate residues (Table 10.1). These findings suggest that defining the chain length is important to optimize stain removal efficiency in both short-chain polyP and ultraphosphate. Although ultraphosphate is a better stain remover than short-chain polyP, its efficiency in preventing stain deposition was comparable to that of short-chain polyP.

Koyasu et al. (2014) also reported that ultraphosphate removes stains from detached natural human teeth and prevents stain deposition at a high efficiency. This stain control activity is also effective in a glass ionomer cement, which is widely used as dental cement. Since polyP group including ultraphosphate has a strong affinity for calcium, ultraphosphate could bind to the glass ionomer cement whose basic ingredient is calcium aluminosilicate and could replace stains that were bound to its surface. They also demonstrated that both stain removal and prevention of stain deposition activity of ultraphosphate were superior to that of various agents that have been used as stain removers in toothpastes including sodium phytic acid and sodium malic acid.

10.14 Enhancement of Bleaching Activity of Peroxide by PolyP

For teeth whitening, bleaching agents such as hydrogen peroxide and carbamide peroxide are widely used. We found that polyP enhances the bleaching activity of hydrogen peroxide and carbamide peroxide. To evaluate the effect of polyP on bleaching agents, heavily colorized hydroxyapatite powders were prepared by sequential treatments of 1 % gelatin solution, concentrated tea and coffee solution (5 g instant coffee granules and four tea bags in 110 ml of water), and artificial saliva (20 mM HEPES-KOH pH 7.0, 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4). In addition

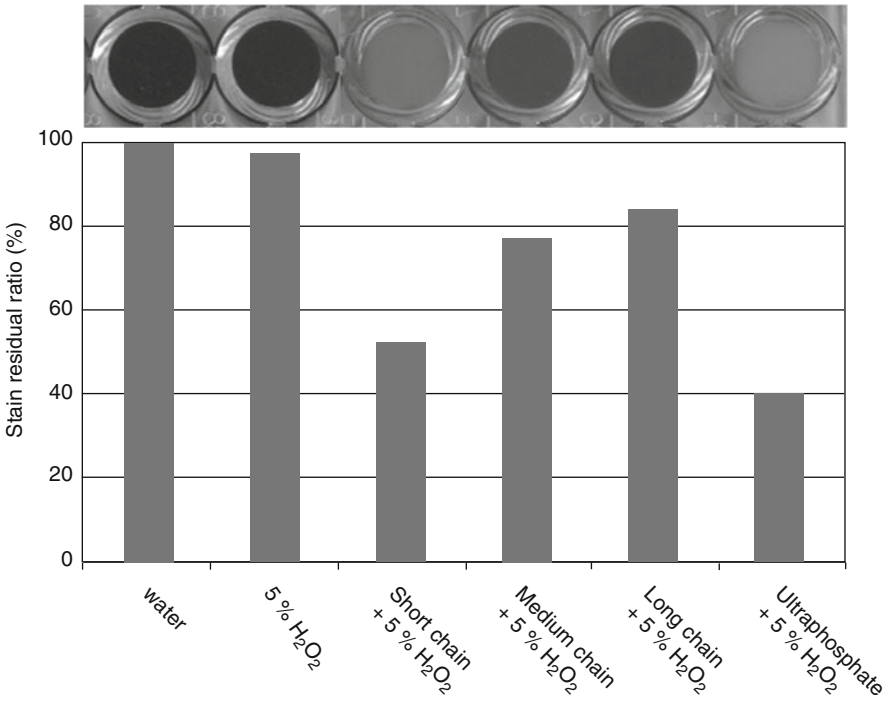

Fig. 10.12 Enhancement of hydrogen peroxide bleaching activity by polyP. Stain residual ratio was calculated in terms of a relative value as described in the legend of Table 10.1

to this colorized hydroxyapatite powder, 1 w/v% of polyP of various chain lengths and sodium ultraphosphate were mixed with 5 % hydrogen peroxide, and the whitening efficiency was examined using the same method as described in Sect. 10.11. As shown in Fig. 10.12, the whitening efficiency of hydrogen peroxide with short-, medium-, and long-chain polyP and ultraphosphate was about 14.8, 7.1, 5.1, and 18.5 times higher than that of hydrogen peroxide alone, respectively. These findings suggest that whitening efficiency was dramatically improved by the combination of polyP and hydrogen peroxide.

References

Akita S, Akino K, Imaizumi T et al (2006) The quality of pediatric burn scars is improved by early administration of basic fibroblast growth factor. J Burn Care Res 27:333–338

Averbuch-Pouchot MT, Durif A (1996) Topics in phosphate chemistry. World Scientific Publishing Co. Pte. Ltd., Singapore

Baig AA, Kozak KM, Cox ER et al (2002) Laboratory studies on chemical whitening effects of a sodium hexametaphosphate dentifrice. J Clin Dent 13:19–24

Gray MJ, Wholey WY, Wagner NO et al (2014) Polyphosphate is a primordial chaperone. Mol Cell 53:689–699

Hacchou Y, Uematsu T, Ueda Y et al (2007) Inorganic polyphosphate: a possible stimulant of bone formation. J Dent Res 86:893–897
Halleen JM, Räisänen S, Salo JJ et al (1999) Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate acid phosphatase. J Biol Chem 274:22907–22910
Harada K, Itoh H, Kawazoe Y et al (2013a) Polyphosphate-mediated inhibition of tartrate-resistant acid phosphatase and suppression of bone resorption of osteoclasts. PLoS One 8:e78612
Harada K, Shiba T, Doi K et al (2013b) Inorganic polyphosphate suppresses lipopolysaccharide-induced inducible nitric oxide synthase (iNOS) expression in macrophages. PLoS One 8:e74650
Hayman AR, Jones SJ, Boyde A et al (1996) Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disrupted endochondral ossification and mild osteopetrosis. Development 122:3151–3162
Kawazoe Y, Shiba T, Nakamura R et al (2004) Induction of calcification in MC3T3-E1 cells by inorganic polyphosphate. J Dent Res 83:613–618
Kawazoe Y, Katoh S, Onodera Y et al (2008) Activation of the FGF signaling pathway and subsequent induction of mesenchymal stem cell differentiation by inorganic polyphosphate. Int J Biol Sci 4:37–47
Koyasu M, Shiba T, Kawazoe Y et al. (2014) Ultraphosphate, a potent stain control agent that is effective for both stain removal and prevention of stain deposition. Dent Mater J 33:1–9
Moreno-Sanchez D, Hernandez-Ruiz L, Ruiz FA et al (2012) Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. J Biol Chem 287:28435–28444
Morita K, Doi K, Kubo T et al (2010) Enhancement initial bone regeneration with inorganic polyphosphate-adsorbed hydroxyapatite. Acta Biomater 6:2808–2815
Müller F, Mutch NJ, Schenk WA et al (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell 139:1143–1156
Murakami S (2007) Periodontal regeneration by FGF-2. Clin Calcium 17:109–115
Omelon S, Georgiou J, Henneman ZJ et al (2009) Control of vertebrate skeletal mineralization by polyphosphates. PLoS One 4:e5634
Ruiz FA, Lea CR, Oldfield E et al (2004) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 279:44250–44257
Segawa S, Fujita M, Konishi H et al (2011) Probiotic-derived polyphosphate enhances the epithelial barrier function and maintains intestinal homeostasis through integrin-p38 MAPK pathway. PLoS One 6:e23278
Shiba T, Nishimura D, Kawazoe Y et al (2003) Modulation of mitogenic activity of fibroblast growth factors by inorganic polyphosphate. J Biol Chem 278:26788–26792
Shiba T, Takahashi Y, Uematsu T et al (2004) Effect of inorganic polyphosphate on periodontal regeneration. Key Eng Mater 254–256:1119–1122
Shiba T, Saitoh A, Okada K et al (2015) Oral stain remover and oral composition. US patent, US 8,992,894 B2
Tsutsumi K, Saito N, Kawazoe Y et al (2014) Morphogenetic study on the maturation of osteoblastic cell as induced by inorganic polyphosphate. PLoS One 9:e86834
Yamaoka M, Uematsu T, Shiba T et al (2008) Effect of inorganic polyphosphate in periodontitis in the elderly. Gerodontology 25:10–17
Yang S, Takahashi N, Yamashita T et al (2005) Muramyl dipeptide enhance osteoclast formation induced by lipopolysaccharide, IL-1 alpha, and TNF-alpha through nucleotide-binding oligomerization domain 2-mediated signaling in osteoblasts. J Immunol 175:1956–1964
Yuan Q, Kubo T, Doi K et al (2009) Effect of combined application of bFGF and inorganic polyphosphate on bioactivities of osteoblast and initial bone regeneration. Acta Biomater 5:1716–1724
Zaidi M, Moonga B, Moss DW et al (1989) Inhibition of osteoclastic acid phosphatase abolishes bone resorption. Biochem Biophys Res Com 159:68–71

Inorganic Polyphosphate in Blood Coagulation

11

Stephanie A. Smith and James H. Morrissey

Abstract

Polyphosphate (polyP) was recently discovered to be stored in a subset of the secretory granules of human platelets (the blood cell that supports formation of clots) and to be secreted upon activation of these cells. It is also present in other human cell types and is present in infectious microorganisms. Work from our laboratory and others has now shown that polyphosphate is a novel, potent modulator of blood clotting that likely plays roles in hemostasis, thrombosis, inflammation, and the host response to pathogens. Polyphosphate acts at multiple points in the coagulation cascade, providing a template for initiation of the contact pathway of clotting, enhancing the activation of factor V (a critical cofactor in clotting whose accelerated activation results in an earlier thrombin burst), and markedly enhancing the rate of activation of factor XI by thrombin (resulting in marked amplification of thrombin generation). Polyphosphate also acts on the formation and degradation of fibrin by becoming incorporated into polymerizing fibrin fibrils (rendering them thicker and obscuring the binding sites for fibrinolytic proteins, which in turn delays clot degradation). Therapeutic agents targeting polyphosphate may have the potential to limit thrombosis with fewer hemorrhagic complications than conventional anticoagulant drugs that target essential proteases of the blood-clotting cascade.

S.A. Smith • J.H. Morrissey (✉)
Department of Biochemistry, University of Illinois at Urbana-Champaign,
323 Roger Adams Laboratory MC-712, 600 South Mathews Avenue,
Urbana, IL 61801, USA
e-mail: jhmorris@illinois.edu

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_11

11.1 Introduction

In 2004, Ruiz et al. (2004) first reported that human platelet dense granules contain an abundant store of inorganic polyphosphate (polyP). Because platelets are important contributors to the prevention of bleeding, this discovery prompted us to investigate possible roles for polyP in blood coagulation. In 2006 we reported that platelet polyP is a potent modulator in the production and removal of blood clots (Smith et al. 2006). Recent studies from our lab and others have now shown that both platelet and microbial polyP are modulators of the blood-clotting cascade. PolyP or suitable derivatives might therefore have utility as hemostatic agents to treat bleeding. Recent work also has implicated polyP as contributing to host-pathogen interactions via its ability to trigger the contact pathway (also known as the kallikrein-kinin pathway). PolyP blockers may therefore have utility as novel antithrombotic/anti-inflammatory agents with reduced bleeding side effects compared with conventional anticoagulant drugs that target the final common pathway of blood clotting.

11.2 Overview of Hemostasis

Blood consists of a suspension of cells (erythrocytes, leukocytes, and platelets) within a highly proteinaceous fluid (plasma). Under normal physiologic conditions, blood flows as a liquid through the vascular system, but in response to injury, blood transforms into a solid that is able to obstruct the breach and prevent further blood loss. The process by which blood loss is prevented is *hemostasis*.

Hemostasis involves a complex interplay between the cells lining the blood vessels (endothelial cells), subendothelial proteins within the vascular wall, platelets, and soluble proteins in the blood. When the blood vessel is healthy and intact, the endothelial cell membrane contains proteins and phospholipids that limit the hemostatic reactions and help maintain blood in a liquid state. The endothelium also secretes inhibitory substances that keep platelets inactive (Krisinger and Conway 2013). When an injury occurs (Fig. 11.1), the endothelial cell becomes activated and changes its surface, expressing proteins and lipids that enhance hemostatic reactions. Injury to the blood vessel also exposes important molecules in the subendothelium that were previously sequestered from the flowing blood. These include collagen, von Willebrand factor (vWF), and tissue factor (TF) (Krisinger and Conway 2013). The binding of collagen and/or vWF to constitutively expressed receptors on the platelet surface causes tethering of platelets to the injury site (adherence) and initiates intracellular signaling processes that result in profound changes in the nature of the platelet (activation) (Bennet 2013). The interaction between subendothelial TF and coagulation proteins in the plasma initiates a cascade of enzymatic reactions that ultimately result in formation of an insoluble fibrin clot in a process known as *coagulation* (White et al. 2013).

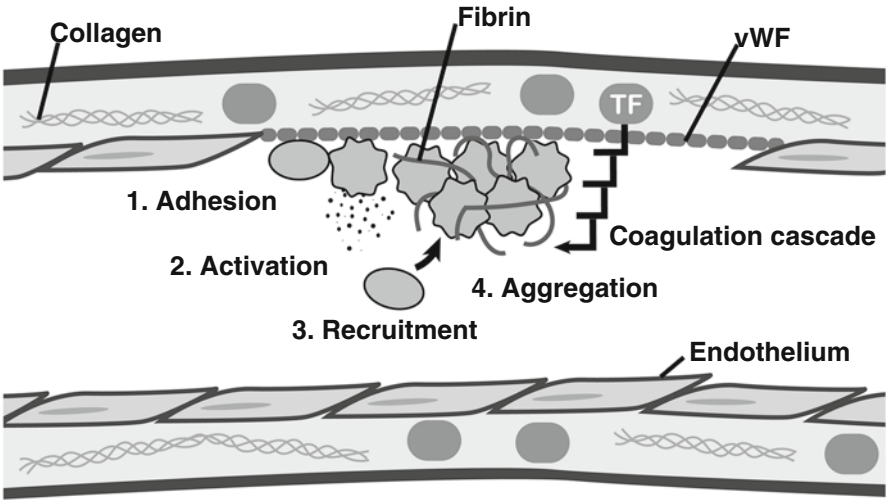

Fig. 11.1 Interactions between platelets, coagulation, and the vascular wall. When an injury occurs, proteins in the vascular wall such as collagen, von Willebrand Factor (vWF), and tissue factor (TF) are exposed to the blood, leading to the following events: (*1*) Adhesion: platelets exposed to vWF are bound to the injury site. (*2*) Activation: binding of platelets to exposed collagen causes changes in platelet structure and surface proteins and results in release of granule contents that activate other platelets. (*3*) Recruitment: new platelets are recruited to the injury site by substances released from granules and by vWF. (*4*) Aggregation: activated platelets bind to one another via fibrinogen bridges and form a platelet plug. In conjunction with these platelet-vessel wall interactions, the exposure of TF initiates the plasma coagulation cascade, ultimately resulting in the formation of fibrin which stabilizes the growing clot

11.3 Platelets

Platelets are anucleate cells but contain an actin cytoskeleton and secretory granules. Their roles in hemostasis include localizing some of the coagulation enzyme-substrate complexes, secreting a variety of proteins that participate in the hemostatic process, and providing a scaffold to support adherence of the fibrin clot to the injury site. Platelets circulate in an inactive state, but when an injury occurs exposing subendothelial collagen, platelets become activated. Activation leads to drastic changes in the platelet, including exposure of a procoagulant membrane surface, rearrangement of cytoskeletal structure to allow for better interactions with other cells, exposure of receptors on the cell surface, and secretion of granule contents. Platelets have multiple granule types: alpha granules contain proteins, dense granules contain small molecules, and lysosomes contain hydrolytic enzymes similar to those in leukocytes (Bennet 2013).

Activation of a few platelets causes release of proteins that recruit new platelets to the site of injury (Bennet 2013). Platelets are also captured by vWF that is released from storage granules in activated endothelial cells (Krisinger and Conway 2013). These newly arrived platelets are then activated by substances released from

previously activated platelets (e.g., adenosine diphosphate (ADP) and thromboxane A_2) and by thrombin produced in the coagulation cascade (see below). Activated platelets also express a high-affinity receptor for fibrinogen on their surface. Fibrinogen binding to these receptors acts as a bridge between two adjacent platelets, tethering them to one another in a process called *aggregation*. Consequently, platelets are both adhered to the wall of the injured vessel and to one another, causing a "platelet plug" that closes the injury site (Bennet 2013).

11.4 Coagulation

Coagulation consists of a cascade of steps where enzymes cleave proenzyme substrates [generally referred to as *factors* (F) and designated with a roman numeral, e.g., FVII] to generate the next enzyme (designated with a lowercase "a," e.g., FVIIa) in the cascade. Many of the steps in the coagulation cascade are enhanced when the enzyme is bound to its procoagulant regulatory protein (cofactor) or inhibited by other regulatory proteins within the plasma (inhibitors). The cascade steps are also greatly facilitated by the proper colocalization of the various protein participants. Some steps employ calcium-dependent localization on a membrane surface containing anionic lipids (such as an activated platelet or endothelial cell), while other steps use a template mechanism where the participants are colocalized on a polymeric anionic surface such as polyP (White et al. 2013).

In hemostasis, the coagulation cascade (Fig. 11.2) is initiated in vivo via the tissue factor pathway when FVIIa in the blood is exposed to the integral membrane protein, TF, in the vascular wall. The TF-FVIIa complex then activates small amounts of FX to FXa. The components of this step in the cascade are inhibited by tissue factor pathway inhibitor (TFPI). The TF-FVIIa complex also activates FIX to FIXa. The generated FXa binds to its cofactor, FVa, to form the FXa-FVa complex, which subsequently cleaves prothrombin and generates a small amount of the central enzyme in coagulation, thrombin. The generated thrombin is available for activation of platelets (see above) and for activation of cofactors, FV and FVIII, as well as the inhibitor, protein C. FVa and FVIIIa are inactivated by activated protein C (APC) owing to proteolysis of these cofactors by APC. FXa and thrombin are also inhibited by antithrombin (AT) in complex with sulfated proteoglycans on the endothelial cell surface (Chung et al. 2013).

The thrombin generated in these early steps can also feedback to activate FXI to FXIa, which in turn activates more FIX to FIXa. The FIXa binds to the activated platelet membrane surface along with its cofactor, FVIIIa. The FIXa-FVIIIa complex then cleaves more FX to FXa, which in turn complexes with its cofactor, FVa, and generates more thrombin (Chung et al. 2013).

Thrombin removes two small peptides from the plasma protein, fibrinogen, generating soluble fibrin. The removal of these peptides exposes binding sites that are complementary to other portions of the fibrin molecule. Once enough thrombin has been generated to convert fibrinogen into soluble fibrin, spontaneous polymerization occurs, forming large insoluble fibrin polymers, resulting in a fibrin clot.

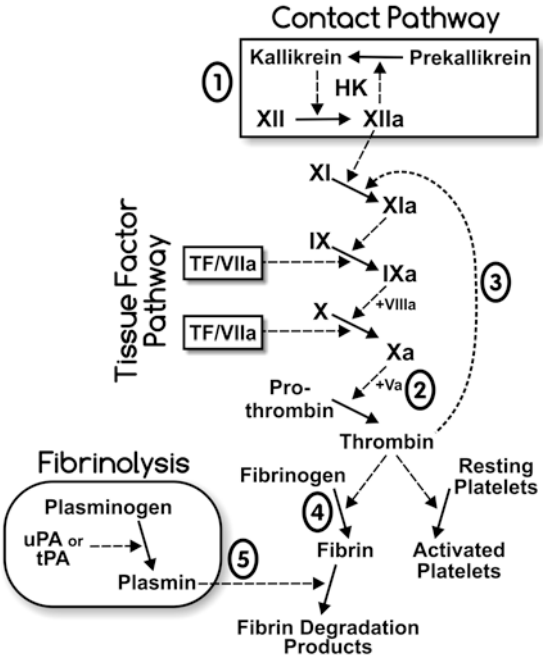

Fig. 11.2 Blood coagulation and fibrinolysis. In hemostasis, the coagulation cascade is initiated via the tissue factor (TF) pathway when FVIIa in the blood is exposed to TF in the vascular wall. The TF-FVIIa complex then activates downstream proteins in the cascade, ultimately leading to the formation of fibrin. In thrombosis, the coagulation cascade may also be initiated via the contact pathway when FXII autoactivates on an anionic surface followed by reciprocal activation of FXII and prekallikrein in the presence of high MW kininogen (HK). The burst of FXIIa activates FXI, leading to propagation of the clotting cascade. In fibrinolysis, plasminogen activators cleave plasminogen to produce plasmin, which breaks down the fibrin clot to soluble degradation products. PolyP acts at multiple points in these cascades. It: (*1*) provides the surface for initiation of the contact pathway; (*2*) enhances the activation of FV to FVa, resulting in an earlier thrombin burst; (*3*) markedly accelerates the back activation of FXI by thrombin; (*4*) is incorporated into the polymerizing fibrin, altering its structure; and (*5*) delays fibrinolysis. Long-chain polyP supports all five of these actions, while platelet-size polyP is most effective in modulating actions *2*, *3*, and *5*

Thrombin also activates FXIII to FXIIIa, a transglutaminase that modifies the structure of the fibrin polymers, resulting in cross-linking between polymers and therefore a stronger clot (Chung et al. 2013).

11.5 The Contact Pathway

An alternative way to initiate the clotting cascade for production of FXIa is the contact pathway. This cascade is activated when FXII localizes on an anionic surface and autoactivates to generate FXIIa. In the classic waterfall model of blood clotting, FXI is then activated by FXIIa. These enzymes are primarily inhibited by C1-inhibitor,

which also inhibits components of the complement system. However, activation of FXI by FXIIa is clearly irrelevant to hemostasis, since severe FXII deficiency is not associated with a bleeding tendency, while severe FXI deficiency is (Seligsohn 2009). This pathway to production of FXIa is, however, important in coagulation of blood when it comes into contact with artificial surfaces (such as in a blood collection tube). Recent evidence suggests also that activation of the contact pathway *is* important for abnormal clot formation that occurs in pathologic conditions that causes inappropriate obstruction of blood vessels (referred to as thrombosis). Disease conditions such as stroke and heart attack are often consequences of thrombosis.

The contact pathway also involves the generation of enzymes that are important in inflammatory processes. FXIIa activates prekallikrein to kallikrein, which is a critical enzyme responsible for release of bradykinin (BK) via proteolysis from high molecular weight (MW) kininogen. Bradykinin, an inflammatory mediator, causes dilation of the blood vessel, swelling, and fluid leakage that are the hallmarks of an inflammatory response. Both FXIIa and kallikrein can also activate urokinase plasminogen activator (uPA), which initiates the fibrinolysis cascade.

11.6 Fibrinolysis

As part of the healing process, the clot needs to be removed to allow for restoration of flow to the previously injured blood vessel. The degradation of the fibrin clot is called *fibrinolysis*. Activated endothelial cells release the enzyme tissue plasminogen activator (tPA) and the contact pathway generates uPA, either of which cleaves the plasma protein plasminogen, activating it to the central enzyme of fibrinolysis, plasmin. Plasmin then degrades the fibrin clot into multiple soluble fragments that can be cleared from the vasculature. Plasminogen activators are directly inhibited by plasminogen activator inhibitor 1 (PAI-1) and indirectly inhibited by thrombin activatable fibrinolysis inhibitor (TAFI). TAFI acts by removing the terminal lysines from the fibrin, which decreases the ability of plasmin to bind to and degrade the fibrin (Mutch and Booth 2013).

11.7 PolyP in Platelets

The dense granules of human platelets contain an abundant store of polyP (Ruiz et al. 2004). These granules share many properties with acidocalcisomes from other organisms: they are spherical organelles that are acidic (Dean et al. 1984), electron-dense (White 1969), and contain metal ions such as Ca^{2+} and Mg^{2+} along with inorganic phosphates (Fukami et al. 1980). PolyP in platelet dense granules is both far less heterodisperse and shorter in length than microbial polyP, ranging in length from about 60 to 100 phosphate units (Ruiz et al. 2004; Muller et al. 2009). Extracts of platelets yielded 0.74 ± 0.08 nmol polyP per 10^8 platelets (Ruiz et al. 2004) (the number expected to be found in approximately 500 μL of blood). The concentration of polyP in the platelet is about 1.1 mM, with an intragranular concentration of around 130 mM (Ruiz et al. 2004). Upon platelet activation, polyP is secreted from

dense granules in conjunction with the other granule contents (Ruiz et al. 2004; Muller et al. 2009). Human patients with defects in platelet dense granules also have reduced levels of polyP in their platelets (Hernández-Ruiz et al. 2009). Although the synthetic pathways responsible for the presence of polyP in platelets are not known, studies of mice missing the gene encoding the enzyme, inositol hexakisphosphate kinase 1 (Ip6k1) indicated that their platelets had tenfold lower polyP levels than normal. These mice also had compromised hemostasis and were protected against experimentally induced thrombosis (Ghosh et al. 2013).

11.8 PolyP in Other Blood Cell Types

Mast cells and basophils also contain secretory granules, a subset of which shares characteristics with acidocalcisomes (Moreno-Sanchez et al. 2012). These acidocalcisome-like secretory granules in mast cells and basophils contain abundant polyP, with polymer lengths very similar to those found in platelets (about 60–100 phosphates long). Mast cell polyP is localized in serotonin, but not in histamine-containing granules, and is released when these cells secrete their granule contents (Moreno-Sanchez et al. 2012). Notably, platelet dense granules also contain serotonin. PolyP has also been reported in human red blood cells as a component of the Ca^{2+}-ATPase pump (Reusch et al. 1997; Reusch 2000).

11.9 PolyP and the Blood Vessel Wall

The vessel wall is an important contributor to the hemostatic process. Molecules within the vascular wall initiate both coagulation and platelet responses. The endothelium participates in regulating both platelet function and some coagulation reactions. In particular, endothelial functions are important in limiting the hemostatic process to the site of injury. The vascular wall also contains a smooth muscle that regulates vascular wall tone, affecting the diameter of the vessel and consequently the flow and pressure within the vessel.

11.10 PolyP and vWF

The function of the multimeric protein, vWF, is to capture platelets in the lumen of blood vessels, bringing them to the site of injury and tethering them to the breach. Recent work suggests that vWF may also be a polyP-regulated protein. PolyP was identified in vWF isolated from human plasma, and polyP appears to interact with vWF in a dose-dependent manner (Montilla et al. 2012). Treatment of purified vWF with exopolyphosphatase reduces the functionality of the protein in in vitro assays (Montilla et al. 2012). Further, there is a significant correlation between the levels of polyP and that of vWF in human patients with inherited deficiency of vWF (Montilla et al. 2012).

11.11 PolyP and Endothelial Cells

Recent studies have evaluated the interactions between polyP and endothelial cells, where it appears to have potent proinflammatory effects. PolyP of the size released from platelets increases both barrier permeability and apoptosis in cultured human umbilical vein endothelial cells (Bae et al. 2012). PolyP also amplifies histone H4- and high mobility group box 1-mediated signaling in endothelial cells. These effects are dependent on P2Y1 and RAGE (receptor for advanced glycation end products) receptor activity (Dinarvand et al. 2014), mediated through the NF-kB pathway (Bae et al. 2012), and inhibited when mammalian target of rapamycin (mTOR) signaling is blocked (Hassanian et al. 2015). PolyP also upregulates endothelial expression of cell-surface associated adhesion molecules that bind leukocytes and platelets to the surface of endothelial cells (Bae et al. 2012). Interestingly, these proinflammatory effects of polyP on endothelial cells are inhibited by APC both in vitro and in vivo (Dinarvand et al. 2014; Bae et al. 2012).

PolyP also has profound effects on the vascular wall via the mediator bradykinin, which is produced in response to polyP-dependent activation of the contact pathway (Muller et al. 2009) (see below). Bradykinin is a potent vasodilator that also decreases endothelial barrier function, leading to fluid leakage. These effects are mediated through bradykinin receptors (BR) on the endothelial cell surface. When polyP is injected into the skin of mice, it also induces vascular leakage in a FXII- and bradykinin-receptor-dependent manner (Muller et al. 2009; Smith et al. 2012). When polyP is injected into the peritoneal cavity in a mouse model, it causes leakage of fluid out of the vasculature and into the cavity, indicating a loss of barrier function (Bae et al. 2012). Intraperitoneal injection induces a lethal, systemic reduction in blood pressure that is also dependent on FXII and bradykinin (Muller et al. 2009).

See Chap. 14 for a more thorough discussion of the proinflammatory effects of polyP.

11.12 Procoagulant PolyP

PolyP of the size secreted by activated platelets acts at multiple steps in the clotting cascade to influence the rate of thrombin generation (Fig. 11.2): it enhances the conversion of FV to FVa, it greatly accelerates FXI activation, and it strongly antagonizes the anticoagulant activity TFPI (Smith et al. 2010; Wood et al. 2013). Thus, an important effect of polyP on blood clotting is accelerating the rate of thrombin generation, shortening the time to the thrombin burst (Smith et al. 2006).

11.13 PolyP Accelerates Activation of FV

FVa occupies a central role in the clotting cascade as the cofactor for FXa that is essential for enzymatic cleavage of prothrombin to generate thrombin. PolyP of the size secreted by activated platelets accelerates the rate of FV activation by multiple

enzymes. It has long been thought that thrombin was the primary enzyme responsible for activating FV, because the production of FVa by either FXa or FXIa was very slow and therefore likely not of physiologic relevance. Recent studies though have indicated that the presence of polyP of the size released by platelets markedly enhances the rate of generation of FVa by all three enzymes (Choi et al. 2014; Smith et al. 2006, 2010).

11.14 PolyP Accelerates Activation of FXI

It has long been known that thrombin can activate FXI (Naito and Fujikawa 1991; Gailani and Broze 1991) in a feedback reaction that was proposed to lead to amplification of thrombin generation, potentially explaining the difference in bleeding phenotype between deficiency of FXII and FXI. However, the highly unfavorable kinetics of this reaction made it unlikely to be significant in vivo (Pedicord et al. 2007; Scott and Colman 1992). We recently discovered that platelet-sized polyP is a highly potent cofactor for the activation of FXI by thrombin (Choi et al. 2011), accelerating the rate of this reaction some 3,000-fold (Geng et al. 2013; Choi et al. 2011). PolyP also markedly accelerates autoactivation of FXI (Choi et al. 2011). Platelet polyP may therefore be the "missing" cofactor that explains the otherwise confusing role of FXI in hemostasis.

11.15 PolyP Antagonizes TFPI

PolyP completely abrogates the anticoagulant function of TFPI in plasma-clotting assays (Smith et al. 2006), and polyP secreted by platelets strongly inhibits TFPI function (Smith et al. 2006; Muller et al. 2009). It has been shown that any FXa that is already bound to its protein cofactor, FVa, is resistant to inhibition by TFPI, especially in the presence of prothrombin (Mast and Broze 1996). The ability of polyP to enhance the rate of generation of FVa may accelerate the protection of newly generated FXa from inhibition by TFPI, in part explaining the abrogation of the inhibitory effects of TFPI. On the other hand, it has also been shown that TFPI can still inhibit FXa in complex with FVa, provided that the FVa is only partially activated as is observed when FV is activated by FXa or when partially activated FV is released from activated platelets (Wood et al. 2013).

11.16 PolyP Initiates the Contact (Kallikrein-Kinin) Pathway

The contact pathway (Fig. 11.3) is triggered when prekallikrein, FXII, and high molecular weight kininogen assemble on an artificial surface or anionic polymer. This results in both autoactivation of FXII and also the reciprocal activation of prekallikrein by FXIIa and of FXII by kallikrein. FXIIa then activates FXI to FXIa, and kallikrein cleaves kininogen to produce bradykinin.

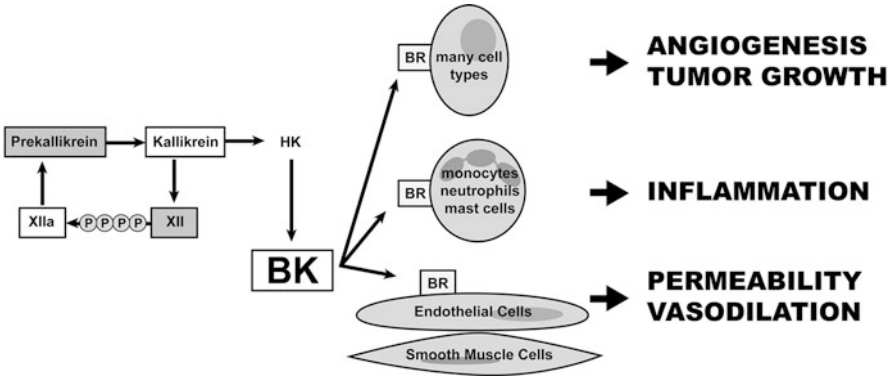

Fig. 11.3 The contact pathway and bradykinin. Activation of FXII on an anionic surface or template (such as polyP) leads to formation of the enzyme kallikrein, which cleaves high molecular weight kininogen (*HK*) to produce the vasoactive peptide bradykinin (*BK*). BK binds to bradykinin receptors (*BR*) on the surface of various cell types, triggering a variety of proinflammatory responses

Although dispensable for hemostasis, the contact pathway is thought to contribute to some types of thrombosis. Epidemiologic studies have shown that elevated FXI correlates with risk of ischemic stroke and venous thromboembolism (He et al. 2012). Further, FXII deficiency protects against arterial and venous thrombosis in multiple animal models (Gailani and Renné 2007; Müller and Renné 2008), while humans with severe FXI deficiency are also protected against some types of thrombosis (Seligsohn 2009; Salomon et al. 2011).

The best-known activators of the contact pathway are artificial surfaces such as glass, clay, and diatomaceous earth. The true (patho)physiologic activator of the contact pathway has not been definitively determined, but candidates include extracellular nucleic acids (Kannemeier et al. 2007), misfolded proteins (Maas et al. 2008), specific microbial proteins (Nickel and Renné 2012), and polyP (Smith et al. 2006, 2010; Muller et al. 2009).

Long-chain polyP (of the length commonly found in microorganisms) is a highly potent activator of the contact pathway both in vitro and in vivo (Smith et al. 2006; Muller et al. 2009). It binds with high affinity to multiple proteins in the contact pathway (Smith et al. 2006, 2010; Choi et al. 2010). Administering high levels of polyP intravenously in mice causes lethal pulmonary embolism, while FXII-deficient mice, or mice receiving an inhibitor of FXIIa, survive (Muller et al. 2009). Long-chain polyP is therefore thrombogenic in vivo in a FXII-dependent manner (Muller et al. 2009). A recent report described the appearance of long-chain polyP on the surface of prostate cancer cells and prostasomes (Nickel et al. 2015). This polyP is procoagulant in vitro and in vivo in a manner that is dependent on the contact pathway, suggesting that cell-surface polyP expression is important in the development of thrombotic complications in prostate cancer (Nickel et al. 2015).

Alternatively, polyP of the size released by activated platelets has poor (but detectable) ability to activate clotting via the contact pathway (Smith et al. 2010).

Several reports have described the ability of activated human platelets to weakly trigger the contact pathway in a FXII-dependent manner (Caen and Wu 2010). These findings are consistent with the concept that platelets are good at accelerating clotting reactions but poor at initiating them.

11.17 PolyP Enhances the Procoagulant Activity of Histones

Since the discovery that neutrophils actively release nucleosomal material (also known as neutrophil extracellular traps or NETs) as part of the inflammatory process (Brinkmann et al. 2004), interest in the possible procoagulant activity of nucleic acids and histones has intensified. NETs promote thrombus formation and histones H4 and H3 directly induce platelet aggregation (Fuchs et al. 2010). Histones also enhance thrombin generation in vitro when polyP is present or in the presence of platelets in a polyP-dependent manner (Semeraro et al. 2011).

11.18 Factor Seven Activating Protease (FSAP)

FSAP is a broad-spectrum serine protease of unclear physiologic relevance that can activate, among others, uPA, FVII, fibrinogen, and high molecular weight kininogen. PolyP is able to support the autoactivation of FSAP, but this effect may require long polymers (Muhl et al. 2009). PolyP of the size in platelets enhances the in vitro activation of uPA by FSAP two- to threefold, but as other anionic polymers also enhance this reaction (albeit with less potency), this effect may not be specific to polyP (Stavenuiter et al. 2012; Muhl et al. 2009). PolyP enhances the ability of FSAP to induce proliferation of vascular wall smooth muscle cells (Muhl et al. 2009). PolyP also can serve as a cofactor for inhibition of FSAP by PAI-1 (Muhl et al. 2009).

11.19 PolyP and Clot Structure

Fibrin clots formed in the presence of polyP are more turbid, contain thicker fibrin fibrils, are more rigid, and are more resistant to fibrinolysis than are fibrin clots formed in the absence of polyP (Smith and Morrissey 2008b; Mutch et al. 2010) (Fig. 11.4). It appears that polyP may be incorporated directly into fibrin clots, and the polyP effect on clot structure also requires calcium (Smith and Morrissey 2008b). The optimal enhancement of fibrin clot structure requires polymers longer than those released from platelets, at least 300 units in length (Smith et al. 2010). Interestingly, pyrophosphate abrogates the ability of polyP to enhance fibrin clot structure but has no measurable effect on fibrin clots formed in the absence of polyP (Smith et al. 2010). Platelet dense granules also contain substantial quantities of pyrophosphate (Ruiz et al. 2004), but little is known about its function.

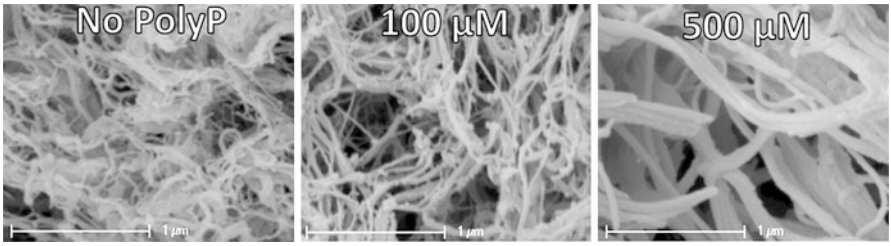

Fig. 11.4 PolyP increases the thickness of fibrin fibrils. PolyP is incorporated into the polymerizing fibrin, resulting in fibrils that are thicker than those formed without polyP. These scanning electron micrographs are of clots formed in vitro by mixing purified human fibrinogen with thrombin in the absence or presence of 100 or 500 µM polyP. *Bar* = 1 µm

11.20 Antifibrinolytic PolyP

In addition to the effects of polyP on the formation of the clot, polyP also delays the degradation of clots in vitro, regardless of whether the fibrinolysis is due to preactivated plasmin or to plasmin that is generated from plasminogen and tPA (Smith and Morrissey 2008b). Interestingly, this resistance to lysis is not merely a function of the thicker fibers that are produced, as most other conditions that increase fibril thickness actually increase susceptibility to fibrinolysis (Smith and Morrissey 2008b).

11.21 PolyP Inhibits Binding of Fibrinolysis Proteins to Fibrin

Forming fibrin clots in the presence of polyP results in inhibition of binding of tPA and plasminogen to fibrin (Mutch et al. 2010), likely due to masking of the C-terminal lysine binding sites for these proteins. Because binding of plasminogen and tPA to partially degraded fibrin is an important step in the activation of plasminogen, polyP reduces the rate of generation of plasmin (Mutch et al. 2010).

11.22 PolyP Causes Earlier Generation of TAFI

Some of the antifibrinolytic effect of polyP is mediated through TAFI. As described above, activated TAFI slows fibrinolysis by removing C-terminal lysine residues from fibrin, which are important in the binding of plasminogen and tPA and accelerate the generation of plasmin. Interestingly, the effect of polyP does not appear to be a function of a direct impact on either the activity or stability of TAFIa. Rather, it likely results from the enhanced generation of thrombin in the presence of polyP. Because TAFI is activated by thrombin, the polyP allows for generation of

TAFIa (and consequently the modification of fibrin by TAFIa) earlier in the course of the coagulation process (Smith et al. 2006).

11.23 PolyP as a Crosstalk Mediator with Inflammation

The appreciation for the interconnectedness between coagulation and inflammation (Esmon et al. 2011) has recently expanded markedly, and much of it is centered on platelets as novel immune mediator cells (Morrell et al. 2014). PolyP appears to be one of the important molecules that mediates this connection. See Chap. 14 for a more thorough discussion of polyP as an inflammatory mediator.

11.24 PolyP and the Complement Cascade

Although the complement cascade is a vital component of the innate immune response, there are multiple areas of intersection between it and the coagulation and contact pathways. The critical regulator of the complement pathway, factor H, also interacts with thrombomodulin (an endothelial surface protein important in activation of protein C) and vWF (Dzik et al. 2000; Rayes et al. 2014). Both plasmin (Barthel et al. 2012) and TAFIa (Campbell et al. 2002) can degrade C3a and C5a.

Recent reports have indicated that polyP destabilizes C5b-6 and reduces the binding of C5b-7 and C5b-8 to the target membrane, functionally inhibiting the terminal portion of the complement system. PolyP also binds to C1-inhibitor and enhances its activity, which suppresses the complement cascade (Wat et al. 2014). Note that C1-inhibitor also regulates the contact pathway. PolyP additionally binds to factor H, and both C1-inhibitor and factor H are released from activated platelets (Licht et al. 2009).

11.25 PolyP and the Contact Pathway

As discussed previously, the contact pathway is an important contributor to inflammation. Activation of the contact pathway (possibly by long-chain polyP) results in kallikrein-mediated release of bradykinin via proteolysis of high molecular weight kininogen. Bradykinin has potent vasoactive functions; when bradykinin binds to its receptors on the endothelial cell, it causes release of prostacyclin, nitric oxide, and endothelium-derived hyperpolarizing factor, resulting in vasodilation (Björkqvist et al. 2013). In addition to bradykinin generation, kallikrein has been shown to directly activate complement components C3 and C5 (Wiggins et al. 1981; DiScipio 1982), while FXIIa also initiates the classical complement cascade (Ghebrehiwet et al. 1983).

The contact pathway contributes to host responses to infection. FXII deficiency in mice is associated with defective response to pathogens (Frick et al. 2006). Further, multiple microbial contact activators (e.g., bacterial surface proteins (Ben

Nasr et al. 1994, 1996), lipopolysaccharide (Kalter et al. 1983), and teichoic/lipoteichoic acid (Kalter et al. 1983) have been identified. The fact that long-chain polyP (like that found in microorganisms) is a potent activator of the contact pathway, while shorter polyP derived from mast cells and platelets is a much weaker contact activator, suggests that microbial polyP may be an important part of the host response to infection, activating as a pathogen-associated danger molecule.

11.26 Potential as a Therapeutic Drug or Target of Drugs

11.26.1 PolyP as a Hemostatic Agent

Current agents used for bleeding episodes, especially those designed to treat internal bleeding, are primarily based upon recombinant coagulation proteins that are expensive to produce and have relatively short shelf lives once reconstituted. In contrast, polyP is already produced cheaply in industrial quantities and is very stable when stored appropriately. PolyP-based hemostatic agents are therefore an intriguing possibility for treating bleeding disorders. Silica nanoparticles functionalized with polyP have been shown to be more effective than polyP alone in enhancing coagulation in vitro (Kudela et al. 2015). Polyphosphate also enhanced the hemostatic properties of a chitosan-based wound dressing (Ong et al. 2008).

11.26.2 PolyP as a Universal Reversal Agent for Anticoagulants

Anticoagulant agents (commonly referred to in the lay public as "blood thinners") are frequently administered to human patients to prevent the development or recurrence of thrombosis. A variety of anticoagulant drugs are in wide use, including warfarin and its derivatives (vitamin K antagonists which decrease the activities of prothrombin, FVII, FIX, and FX), heparins and heparin-like drugs (which catalyze the inhibitory function of antithrombin), and small-molecule drugs that directly inhibit coagulation enzymes (in particular, FXa or thrombin). PolyP is procoagulant in vitro regardless of the type of anticoagulant presently used (Smith and Morrissey 2008a). PolyP, or polyP-based hemostatic agents, may therefore have utility as reversal agents that can antagonize the action of anticoagulant drugs in situations where increased risk of bleeding is a problem.

11.26.3 PolyP Inhibitors as Antithrombotic Agents

We recently have identified proof-of-principal inhibitors of the procoagulant activities of polyP, including cationic proteins, small molecules, and dendrimers (Smith et al. 2012; Jain et al. 2012). These anti-polyP agents are protective against either arterial or venous thrombosis in mouse models, while having fewer bleeding side effects than heparin. Most of these inhibitors unfortunately are toxic in vivo, making

them unlikely to be useful as drugs, but recent development of a new class of non-toxic, dendrimer-like cationic compounds may have addressed this problem. These molecules are potent inhibitors of polyP, are effective in mouse models at preventing thrombosis, and have markedly reduced tendency to cause bleeding as compared to the commonly employed anticoagulant agent, heparin (Travers et al. 2014).

Conclusions

The recent discovery of polyP in platelets led to a new awareness of its multiple roles in hemostasis, thrombosis, immunity, and inflammation. PolyP is also both a potential drug target and potential therapeutic agent for a variety of serious disorders of hemostasis and inflammation, including bleeding due to trauma or adverse consequences of therapeutics as well as arterial and venous thrombosis. The information included here possibly represents a small portion of the discoveries to come but will provide the foundation for what is likely to be an expanding field.

Acknowledgments The authors' studies were supported by grant R01 HL047014 from the National Heart, Lung, and Blood Institute of the National Institutes of Health. We thank Catherine Baker for assistance with preparing figures for this chapter.

References

Bae JS, Lee W, Rezaie AR (2012) Polyphosphate elicits pro-inflammatory responses that are counteracted by activated protein C in both cellular and animal models. J Thromb Haemost 10(6):1145–1151. doi:10.1111/j.1538-7836.2012.04671.x

Barthel D, Schindler S, Zipfel PF (2012) Plasminogen is a complement inhibitor. J Biol Chem 287(22):18831–18842. doi:10.1074/jbc.M111.323287

Ben Nasr A, Wistedt A, Ringdahl U et al (1994) Streptokinase activates plasminogen bound to human group C and G streptococci through M-like proteins. Eur J Biochem 222(2):267–276

Ben Nasr A, Olsen A, Sjöbring U et al (1996) Assembly of human contact phase proteins and release of bradykinin at the surface of curli-expressing *Escherichia coli*. Mol Microbiol 20(5):927–935

Bennet JS (2013) Overview of megakaryocyte and platelet biology. In: Marder VJ, Aird WC, Bennet JS, Schulman S, White GC, 2nd (eds) Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Lippincott Williams & Wilkins, Philadelphia, pp 341–348

Björkqvist J, Jämsä A, Renné T (2013) Plasma kallikrein: the bradykinin-producing enzyme. Thromb Haemost 110(3):399–407. doi:10.1160/TH13-03-0258

Brinkmann V, Reichard U, Goosmann C et al (2004) Neutrophil extracellular traps kill bacteria. Science (New York, NY) 303(5663):1532–1535. doi:10.1126/science.1092385

Caen J, Wu Q (2010) Hageman factor, platelets and polyphosphates: early history and recent connection. J Thromb Haemost 8(8):1670–1674. doi:10.1111/j.1538-7836.2010.03893.x

Campbell WD, Lazoura E, Okada N et al (2002) Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. Microbiol Immunol 46(2):131–134

Choi SH, Collins JN, Smith SA et al (2010) Phosphoramidate end labeling of inorganic polyphosphates: facile manipulation of polyphosphate for investigating and modulating its biological activities. Biochemistry 49(45):9935–9941. doi:10.1021/bi1014437

Choi SH, Smith SA, Morrissey JH (2011) Polyphosphate is a cofactor for the activation of factor XI by thrombin. Blood 118(26):6963–6970, doi:blood-2011-07-368811 [pii]

Choi SH, Smith SA, Morrissey JH (2014) Polyphosphate accelerates factor V activation by factor XIa. Thromb Haemost 113(2):599–604. doi:10.1160/th14-06-0515

Chung D, Xu W, Davie EW (2013) The blood coagulation factors and inhibitors: their primary structure, complementary DNAs, genes, and expression. In: Marder VJ, Aird WC, Bennet JS, Schulman S, White GC 2nd (eds) Hemostasis and Thrombosis: Basic Principles and Clinical Practice, 6th edn. Lippincott Williams & Wilkins, Philadelphia, pp 110–145

Dean GE, Fishkes H, Nelson PJ et al (1984) The hydrogen ion-pumping adenosine triphosphatase of platelet dense granule membrane. Differences from F_1F_0- and phosphoenzyme-type ATPases. J Biol Chem 259(15):9569–9574

Dinarvand P, Hassanian SM, Qureshi SH et al (2014) Polyphosphate amplifies proinflammatory responses of nuclear proteins through interaction with receptor for advanced glycation end products and P2Y1 purinergic receptor. Blood 123(6):935–945. doi:10.1182/blood-2013-09-529602

DiScipio RG (1982) The activation of the alternative pathway C3 convertase by human plasma kallikrein. Immunology 45(3):587–595

Dzik S, Aubuchon J, Jeffries L et al (2000) Leukocyte reduction of blood components: public policy and new technology. Transfus Med Rev 14(1):34–52, doi:S0887-7963(00)80114-5 [pii]

Esmon CT, Xu J, Lupu F (2011) Innate immunity and coagulation. J Thromb Haemost 9(Suppl 1):182–188. doi:10.1111/j.1538-7836.2011.04323.x

Frick IM, Akesson P, Herwald H et al (2006) The contact system: a novel branch of innate immunity generating antibacterial peptides. EMBO J 25(23):5569–5578. doi:10.1038/sj.emboj.7601422

Fuchs TA, Brill A, Duerschmied D et al (2010) Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A 107(36):15880–15885. doi:10.1073/pnas.1005743107

Fukami MH, Dangelmaier CA, Bauer JS et al (1980) Secretion, subcellular localization and metabolic status of inorganic pyrophosphate in human platelets. A major constituent of the amine-storing granules. Biochem J 192(1):99–105

Gailani D, Broze GJ Jr (1991) Factor XI activation in a revised model of blood coagulation. Science 253(5022):909–912

Gailani D, Renné T (2007) Intrinsic pathway of coagulation and arterial thrombosis. Arterioscler Thromb Vasc Biol 27(12):2507–2513. doi:10.1161/ATVBAHA.107.155952

Geng Y, Verhamme IM, Smith SA et al (2013) Factor XI anion-binding sites are required for productive interactions with polyphosphate. J Thromb Haemost 11(11):2020–2028. doi:10.1111/jth.12414

Ghebrehiwet B, Randazzo BP, Dunn JT et al (1983) Mechanisms of activation of the classical pathway of complement by Hageman factor fragment. J Clin Invest 71(5):1450–1456

Ghosh S, Shukla D, Suman K et al (2013) Inositol hexakisphosphate kinase 1 maintains hemostasis in mice by regulating platelet polyphosphate levels. Blood 122(8):1478–1486. doi:10.1182/blood-2013-01-481549

Hassanian SM, Dinarvand P, Smith SA et al (2015) Inorganic polyphosphate elicits pro-inflammatory responses through activation of the mammalian target of rapamycin complexes 1 and 2 in vascular endothelial cells. J Thromb Haemost 13(5):860–871. doi:10.1111/jth.12899

He R, Chen D, He S (2012) Factor XI: hemostasis, thrombosis, and antithrombosis. Thromb Res 129(5):541–550. doi:10.1016/j.thromres.2011.11.051

Hernández-Ruiz L, Sáez-Benito A, Pujol-Moix N et al (2009) Platelet inorganic polyphosphate decreases in patients with delta storage pool disease. J Thromb Haemost 7(2):361–363. doi:10.1111/j.1538-7836.2008.03238.x

Jain S, Pitoc GA, Holl EK et al (2012) Nucleic acid scavengers inhibit thrombosis without increasing bleeding. Proc Natl Acad Sci U S A 109(32):12938–12943. doi:10.1073/pnas.1204928109

Kalter ES, van Dijk WC, Timmerman A et al (1983) Activation of purified human plasma prekallikrein triggered by cell wall fractions of *Escherichia coli* and *Staphylococcus aureus*. J Infect Dis 148(4):682–691

Kannemeier C, Shibamiya A, Nakazawa F et al (2007) Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. Proc Natl Acad Sci U S A 104(15):6388–6393

Krisinger MJ, Conway EM (2013) Role of the endothelium in hemostasis. In: Marder VJ, Aird WC, Bennet JS, Schulman S, White GC II (eds) Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Lippincott Williams & Wilkins, Philadelphia, pp 569–578

Kudela D, Smith SA, May-Masnou A et al (2015) Clotting activity of polyphosphate-functionalized silica nanoparticles. Angew Chem Int Ed Engl 54(13):4018–4022. doi:10.1002/anie.201409639

Licht C, Pluthero FG, Li L et al (2009) Platelet-associated complement factor H in healthy persons and patients with atypical HUS. Blood 114(20):4538–4545. doi:10.1182/blood-2009-03-205096

Maas C, Govers-Riemslag JW, Bouma B et al (2008) Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation. J Clin Invest 118(9):3208–3218. doi:10.1172/JCI35424

Mast AE, Broze GJ Jr (1996) Physiological concentrations of tissue factor pathway inhibitor do not inhibit prothrombinase. Blood 87(5):1845–1850

Montilla M, Hernandez-Ruiz L, Garcia-Cozar FJ et al (2012) Polyphosphate binds to human von Willebrand factor in vivo and modulates its interaction with glycoprotein Ib. J Thromb Haemost 10(11):2315–2323. doi:10.1111/jth.12004

Moreno-Sanchez D, Hernandez-Ruiz L, Ruiz FA et al (2012) Polyphosphate is a novel pro-inflammatory regulator of mast cells and is located in acidocalcisomes. J Biol Chem 287(34):28435–28444. doi:10.1074/jbc.M112.385823

Morrell CN, Aggrey AA, Chapman LM et al (2014) Emerging roles for platelets as immune and inflammatory cells. Blood 123(18):2759–2767. doi:10.1182/blood-2013-11-462432

Muhl L, Galuska SP, Oorni K et al (2009) High negative charge-to-size ratio in polyphosphates and heparin regulates factor VII-activating protease. FEBS J 276(17):4828–4839. doi:10.1111/j.1742-4658.2009.07183.x

Müller F, Renné T (2008) Novel roles for factor XII-driven plasma contact activation system. Curr Opin Hematol 15(5):516–521. doi:10.1097/MOH.0b013e328309ec85

Muller F, Mutch NJ, Schenk WA et al (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell 139(6):1143–1156

Mutch NJ, Booth NA (2013) Plasminogen activation and regulation of fibrinolysis. In: Marder VJ, Aird WC, Bennet JS, Schulman S, White GC, 2nd (eds) Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Lippincott Williams & Wilkins, Philadelphia, pp 314–333

Mutch NJ, Engel R, Uitte de Willige S et al (2010) Polyphosphate modifies the fibrin network and down-regulates fibrinolysis by attenuating binding of tPA and plasminogen to fibrin. Blood 115(19):3980–3988. doi:10.1182/blood-2009-11-254029

Naito K, Fujikawa K (1991) Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. J Biol Chem 266(12):7353–7358

Nickel KF, Renné T (2012) Crosstalk of the plasma contact system with bacteria. Thromb Res 130(Suppl 1):S78–S83. doi:10.1016/j.thromres.2012.08.284

Nickel KF, Ronquist G, Langer F et al (2015) The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis. Blood 126(11):1379–1389. doi:10.1182/blood-2015-01-622811

Ong SY, Wu J, Moochhala SM et al (2008) Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties. Biomaterials 29(32):4323–4332. doi:10.1016/j.biomaterials.2008.07.034

Pedicord DL, Seiffert D, Blat Y (2007) Feedback activation of factor XI by thrombin does not occur in plasma. Proc Natl Acad Sci U S A 104(31):12855–12860. doi:10.1073/pnas.0705566104

Rayes J, Roumenina LT, Dimitrov JD et al (2014) The interaction between factor H and VWF increases factor H cofactor activity and regulates VWF prothrombotic status. Blood 123(1):121–125. doi:10.1182/blood-2013-04-495853

Reusch RN (2000) Transmembrane ion transport by polyphosphate/poly-(R)-3-hydroxybutyrate complexes. Biochemistry (Mosc) 65(3):280–295

Reusch RN, Huang R, Kosk-Kosicka D (1997) Novel components and enzymatic activities of the human erythrocyte plasma membrane calcium pump. FEBS Lett 412(3):592–596

Ruiz FA, Lea CR, Oldfield E et al (2004) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 279(43):44250–44257

Salomon O, Steinberg DM, Zucker M et al (2011) Patients with severe factor XI deficiency have a reduced incidence of deep-vein thrombosis. Thromb Haemost 105(2):269–273. doi:10.1160/TH10-05-0307

Scott CF, Colman RW (1992) Fibrinogen blocks the autoactivation and thrombin-mediated activation of factor XI on dextran sulfate. Proc Natl Acad Sci U S A 89:11189–11193

Seligsohn U (2009) Factor XI deficiency in humans. J Thromb Haemost 7(Suppl 1):84–87. doi:10.1111/j.1538-7836.2009.03395.x

Semeraro F, Ammollo CT, Morrissey JH et al (2011) Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. Blood 118(7):1952–1961. doi:10.1182/blood-2011-03-343061

Smith SA, Morrissey JH (2008a) Polyphosphate as a general procoagulant agent. J Thromb Haemost 6(10):1750–1756. doi:10.1111/j.1538-7836.2008.03104.x

Smith SA, Morrissey JH (2008b) Polyphosphate enhances fibrin clot structure. Blood 112(7):2810–2816, doi:blood-2008-03-145755 [pii]

Smith SA, Mutch NJ, Baskar D et al (2006) Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci U S A 103(4):903–908, doi:0507195103 [pii]

Smith SA, Choi SH, Davis-Harrison R et al (2010) Polyphosphate exerts differential effects on blood clotting, depending on polymer size. Blood 116(20):4353–4359, doi:blood-2010-01-266791 [pii]

Smith SA, Choi SH, Collins JN et al (2012) Inhibition of polyphosphate as a novel strategy for preventing thrombosis and inflammation. Blood 120(26):5103–5110. doi:10.1182/blood-2012-07-444935

Stavenuiter F, Dienava-Verdoold I, Boon-Spijker MG et al (2012) Factor seven activating protease (FSAP): does it activate factor VII? J Thromb Haemost 10(5):859–866. doi:10.1111/j.1538-7836.2012.04619.x

Travers RJ, Shenoi RA, Kalathottukaren MT et al (2014) Nontoxic polyphosphate inhibitors reduce thrombosis while sparing hemostasis. Blood 124(22):3183–3190. doi:10.1182/blood-2014-05-577932

Wat JM, Foley JH, Krisinger MJ et al (2014) Polyphosphate suppresses complement via the terminal pathway. Blood 123(5):768–776. doi:10.1182/blood-2013-07-515726

White JG (1969) The dense bodies of human platelets: inherent electron opacity of the serotonin storage particles. Blood 33(4):598–606

White II GC, Marder VJ, Schulman S, Aird WC, Bennet JS (2013) Overview of basic coagulation and fibrinolysis. In: Marder VJ, Aird WC, Bennet JS, Schulman S, White II GC, (eds) Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Lippincott Williams & Wilkins, Philadelphia, pp 103–109

Wiggins RC, Giclas PC, Henson PM (1981) Chemotactic activity generated from the fifth component of complement by plasma kallikrein of the rabbit. J Exp Med 153(6):1391–1404

Wood JP, Bunce MW, Maroney SA et al (2013) Tissue factor pathway inhibitor-alpha inhibits prothrombinase during the initiation of blood coagulation. Proc Natl Acad Sci U S A 110(44):17838–17843. doi:10.1073/pnas.1310444110

Influence of Condensed Phosphates on the Physical Chemistry of Calcium Phosphate Solids

12

Sidney Omelon and Wouter Habraken

Abstract

This is not the first summary of the contributions toward understanding calcium phosphate spheres found in biology; however, it describes new options for the possible role of condensed phosphates (pyrophosphate and polyphosphates) in the formation, stability, and sometimes the crystallization of these spheres. The first section will briefly review the inorganic conditions required for mineral nucleation and growth and the industrial use of condensed phosphates as inhibitors of crystal formation. The capacity of condensed phosphates to stabilize spherical, x-ray amorphous, Ca- and P-rich granules in biology will be reviewed, and some biological examples provided. Condensed phosphates spontaneously hydrolyze into inorganic phosphate ions in aqueous environments, but the rate is affected by many factors. One accelerator of condensed phosphate hydrolytic degradation is active phosphatase enzymes; these enzymes introduce the potential for biological control of calcium phosphate crystal nucleation by reducing the nucleation-inhibiting effect of condensed phosphates, while simultaneously increasing the Pi concentration – and therefore phosphate crystal supersaturation. The relationship of these condensed phosphate hydrolysis factors on sample preparation and sample observation and the role phosphatase enzymes for biological phosphate mineral formation will be briefly reviewed for some prokaryotes and eukaryotes in which amorphous and/or crystalline Ca- and P-rich solids have been identified.

S. Omelon (✉)
Department of Chemical and Biological Engineering, University of Ottawa, 161 Louis Pasteur Pvt., Ottawa, ON K1N 6N5, Canada
e-mail: Sidney.Omelon@uOttawa.ca

W. Habraken
Department of Biomaterials, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, Potsdam-Golm 14467, Germany

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_12

12.1 Introduction

The most common crystalline, calcium phosphate biological solid phase is a highly substituted, carbonated calcium phosphate solid, often with a significant fluoride content. Sometimes referred to as "carbonated apatite" (CA) or "carbonated fluorapatite" (CFA), it has been identified within some eukaryotes and is associated with some prokaryotes. Identification and characterization of spherical, x-ray amorphous, Ca- and P-rich solids in biology have fueled more curiosity and supported the theory that an amorphous solid phase contributes to the formation of crystalline C(F)A. The study of the biological mechanisms by which crystalline, or amorphous, Ca- and P-rich solids nucleate within or external to organisms continues to be a field full of more questions than answers. The rules of chemistry and physics must be followed, yet the pathways of calcium and phosphate ions, from components of an organism's food to an intracellular amorphous Ca- and P-rich sphere or an intracellular or extracellular, crystalline Ca- and P-rich solid, continue to beguile those whose attention these questions take. Biological crystals are known to form not only through classical inorganic solution thermodynamics, but they are also generated from aggregations of initially intracellular, labile, amorphous, nano-sized spheres.

Classical solution thermodynamics explains how condensed phosphates (CP) inhibit crystal nucleation and growth from solution, which explains their industrial use to prevent "scaling." In this chapter, CP is used as a general term to describe linear polyphosphates, as both pyrophosphate (PPi: $P_2O_7^{4-}$) and polyphosphate (polyP: $[(PO_3)^-]_n$) inhibit crystal nucleation, but PPi is additionally a component in a wide range of biochemical reactions. Biological processes would also benefit from the effect of CPs on crystal nucleation and growth, as well as its stabilization of amorphous phases. CP formation and destruction also affect phosphate ion activity, which is reduced through condensation of inorganic phosphate ions (Pi) by kinase enzymes. Pi activity is biochemically increased by cleaving Pi from CPs by phosphatase enzymes. As there is evidence for a relationship between the trio of Ca- and P-rich amorphous precursors, phosphatase activity, and biological phosphate crystallization processes, where CP chemistry can affect each trio member, the role(s) of CPs in biological calcium phosphate solid formation may benefit from more investigation.

Understanding the potential role(s) of CP in the formation of amorphous and crystalline solids in prokaryotic and eukaryotic organisms is difficult due to challenges in sample preparation, extraction, and detection techniques that could dissolve or change the nature of the solid and any CP content. It is almost impossible to probe biological microenvironments or tissues in which biological calcium phosphate solids form without affecting them; however, advances in cryo- and in vivo techniques provide exciting options for characterizing the steps in biological calcium phosphate solid formation. Understanding the effects of sample preparation and analysis is useful for data interpretation; purposefully testing and observing these effects have been reported by many to understand the nature of biological calcium phosphate solids. This chapter will provide brief reviews of the phosphate

"minerals" associated with prokaryote and eukaryote life (and death), crystal nucleation and growth, amorphous P-rich solid phases in prokaryotes and eukaryotes, as well as the roles of phosphatases, sample preparation, and analysis on CPs as well as calcium phosphate solid phase transition and identification.

12.2 Phosphate "Minerals" in Biology

A wide range of biologically induced or controlled processes result in the formation of minerals that otherwise would not crystallize from their inorganic geochemical environment. Strictly speaking, many of the phosphate "minerals" produced by life forms are not minerals, because this term is reserved for crystalline solids produced by inorganic processes. This distinguishing characteristic also clarifies the point that biological phosphate crystals are not produced by only inorganic processes. The only true phosphate mineral associated with a biological system is hydroxylapatite, which has been identified as one of the crystals in dental plaque (Legeros 1974). The more common phosphate crystals in biology are C(F)A with a crystalline water component (McConnell 1973; Pasteris et al. 2014) and there are many reviews on the crystal chemistry of this skeletal apatite (Skinner 1989; Brown and Chow 1976; LeGeros 1991; Weiner and Wagner 1998; Rey et al. 2009). Although widely used in the literature, C(F)A is not a true "mineral"; its exact formation mechanism(s) is still not known, and its chemistry is consistently complex.

A possible reason for its complexity could be that C(F)A is sometimes created within an organism, where it is assumed the environment is controlled or sometimes located external to the organism – either within an extracellular matrix that is open or closed to the environment. C(F)A is produced by different bacteria and eukaryotes that differ from the two other major domains of the tree of life (prokaryotes and archaea) because they exhibit compartmentalized volumes. These compartmentalized volumes include mitochondria, microtubules, and other volumes segmented by membranes (Margulis 1981; Pace 2006). Within these vesicles, eukaryotes can generate microenvironments with discreet, controllable, and dynamic chemical compositions, from which phosphate solids that are difficult to predict and characterize are produced. Even though these microenvironments can lead to surprising results, these solids must still follow what we understand of nucleation theory.

12.3 Crystallization Theory for Calcium Phosphates

The concept of mineral saturation is central to crystallization (nucleation, crystal growth, and crystal dissolution) theory. The saturation state (degree of saturation) of a particular mineral (Ω) depends on the temperature-dependent solubility product (K_{sp}) for a mineral and the ion activity product (IAP) of the mineral component ions (α). The IAP is calculated by the chemical activities of the mineral ions, raised to the power of their mineral stoichiometric coefficient. The IAP divided by the K_{sp} estimates the saturation state (Morse 1974) (Eq. 12.1).

$$\Omega_{X_xY_y} = \frac{\left(\alpha_{X^+}\right)^x \left(\alpha_{Y^-}\right)^y}{K_{sp}\left(T\right)} \tag{12.1}$$

The ion chemical activities are proportional to their concentration; the activity coefficient takes into account the non-ideality of the solution. The activity coefficient commonly reduces the ion activity below the value of the total concentration. A crystal or amorphous state may form from a solution with a saturation greater than one, a condition called supersaturation, and will be expected to dissolve when the saturation is less than one (undersaturation). The supersaturation state required for a crystal to nucleate spontaneously from solution is greater than that for a crystal nucleus to grow on a substrate; therefore spontaneous nucleation requires a larger ion activity product. Kinetics also serve a role in nucleation processes and will not be reviewed here.

The K_{sp} and the ion activity product for biological C(F)A are difficult to ascertain, as there are many component ions with varying compositions and possible modifications to the ion activities due to complicated biological environments. To simplify the approach to calcium phosphate nucleation, some investigators have simplified the calculation of saturation state to a value that is proportional to the "free" calcium multiplied by the "free" phosphate ion concentrations: $\left[Ca^{2+}\right] \times \left[Pi\right]$ (Fleisch and Neuman 1961). "Free" ions represent the fraction of ions that are not associated with other species in the system and are therefore free to participate in crystal formation and representative of the activity coefficient. This analogy will be used in this chapter; for simplification, the effect of pH on the speciation of the inorganic phosphate ion will not be discussed.

12.4 Effect of Condensed Phosphates on Calcium Phosphate Saturation

The polymerization of CPs from Pi affects the IAP of calcium phosphate minerals in two ways. Polymerization of Pi ions reduces their concentration, which reduces their activity. CPs also have a strong affinity for multivalent cations, as they are negatively charged polyanions. When complexed with CPs, cations are less "free" to participate in crystal formation, so their activity is reduced (Van Wazer and Campanella 1950). CPs complexed with multivalent cations can also form distinct nanoparticles with some stability (Harold 1963) that are difficult to extract intact (Muhammed 1959). In a closed environment, the theoretical formation of CPs by a kinase enzyme would dramatically reduce the calcium phosphate crystal IAP by reducing the contributions of both the Pi and the Ca^{2+} activities to the CFA saturation state.

The depolymerization of CPs that have formed complexes with Ca^{2+} has the opposite effect on the CFA saturation state. CP depolymerization increases the Pi concentration, and as the CP polymer chain size decreases, the number of calcium

ions that associate with it decreases, while the number of "free" calcium ions increases (Omelon and Grynpas 2011). Both of these effects increase the Pi- and Ca^{2+}-free concentrations and therefore the saturation state of calcium phosphate minerals. If a critical supersaturation is reached, by Ostwald's rule, the least stable solid state, which is often a small, spherical, x-ray amorphous phase, will form. However, if only a fraction of the CPs were degraded, a nascent Ca- and Pi-rich amorphous state could be stabilized by the CPs – further complicating the energy required for calcium phosphate crystallization.

12.5 Inhibition of Crystal Nucleation and Growth by Condensed Phosphates

PolyP is one of many molecules added to aqueous solutions to inhibit the formation of "scale" minerals such as calcium carbonate (calcite) on the interior of industrial water pipe surfaces and/or heat exchanger surfaces. The "surface-active properties" of polyP were first noted in 1939 (Hatch and Rice 1939) and associated with forming a soluble compound with calcium (Gilmore 1937). The solubility of CPs is reduced when they are associated with alkaline earth metals at neutral pH (Rosenberg 1966) as there is a strong affinity between them.

PolyP ions were theorized to inhibit the formation of calcite nuclei and prevent the nuclei from growing; this is termed a "threshold" effect (Hatch and Rice 1945; Darton 1997). PolyP and PPi also adversely affect calcium sulfate dihydrate (gypsum) scale formation on metal heat exchanger surfaces (Amjad 1998), suggesting a CP effect on the calcium ion and crystal nucleation. CPs are not as effective as more recent classes of scale-inhibition agents, as CPs spontaneously hydrolyze into Pi. This hydrolysis is accelerated with increasing temperatures and is affected by pH (McCullough et al. 1956; de Jager and Heyns 1998), temperature (Van Wazer et al. 1955; Ketrane et al. 2009), enzymes in soil (Karl-Kroupa et al. 1957; Dick and Tabatabai 1986), colloidal gels, complexing cations, phosphate concentration, and other species in solution (Van Wazer 1958) and results in the production of phosphate sludge (Tyusenkov and Cherepashkin 2014).

Once freed from covalent phosphoanhydride bonds, CP decomposition increases the calcium phosphate IAP and cannot longer exert the threshold effect. Both of these changes reduce the barrier to the onset of crystal nucleation and growth. Simkiss (1964) reviewed the nucleation inhibition ("crystal poison") power of hexametaphosphate (a term unfortunately used to describe linear polyPs and ring polyPs) with a perspective on biological carbonate and phosphate crystal formation and the possibility that polyP destruction by an enzyme could be a biochemical pathway to crystal nucleation. The resulting components within the local conditions, whether in boiler water, constrained within a vesicle, or within a variety of extracellular spaces, may result in the wide range of observed calcium phosphate solid states in biology.

12.6 Condensed Phosphates and Amorphous Phases

12.6.1 Amorphous, P-Rich Phase Stabilization

PolyP was noted to be a more powerful calcium phosphate mineral nucleation and growth inhibitor than PPi and other phosphoesters (Fleisch and Neuman 1961), and they also have an interesting effect on the amorphous calcium phosphate (ACP) solid phase. ACP does not have defined x-ray diffraction peaks, but is identified with specific infrared (Gadaleta et al. 1996) and Raman (Kazanci et al. 2006) spectroscopy signals. ACP is not the most thermodynamically stable calcium phosphate solid state; consequently it will spontaneously crystallize into apatite (Habraken et al. 2013). Crystallization can be inhibited by PPi, as it stabilizes the solid phase from transforming into apatite (Fleisch et al. 1968; Termine et al. 1970) as does polyP (Francis 1969; Betts et al. 1981).

CP-containing, amorphous, P-rich granules have been theorized to serve a bioavailable P-storage strategy (Harold 1966; Rao et al. 2009; Kulaev et al. 2004) as CP chemistry allows for high, intracellular, P concentrations that do not crystallize. Gal, Weiner, and Addadi recently reviewed the role of amorphous nanosphere particle accretion in biomineralization processes that are first x-ray amorphous and their contribution to crystal growth processes (Gal et al. 2015). A CP component within a Ca- and P-rich complex could remain amorphous, without forming a crystalline calcium phosphate solid for three reasons. Firstly, the P concentration is not as a Pi molecule, but as CP, which is not a component of the IAP for phosphate crystals. Therefore the P concentration as CP species can be extraordinarily high, without the IAP exceeding any calcium phosphate mineral solubility product. This is a problem that concerned Lehninger, as he estimated P (and assumed Pi) concentrations of 0.8 M in mitochondrial Ca- and P-rich, amorphous, electron-dense granules and correctly wondered why phosphate crystals did not spontaneously nucleate at this high P (assumed to be Pi) concentration (Lehninger 1970). Secondly, CPs form complexes with calcium ions, reducing their activity and contribution to the IAP. Lastly, the second valuable CP characteristic is the inhibition of nuclei formation and crystal growth by physical interference with nucleation and adsorption on nascent nuclei, inhibiting crystal growth. However, its inhibitory power is not fully explained by calcium binding, so other effects are theorized to explain the "threshold effect" on the development of a crystalline solid phase.

Although Fleisch and Bisaz looked for polyP because of its "strong inhibitory activity," PPi was observed to inhibit calcium phosphate crystal formation in urine. They theorized that PPi also inhibits crystal formation in bone tissue (Fleisch and Bisaz 1962). The multiplicity of effects of CP on crystallization inhibition suggests that a spherical, amorphous, P-rich granule may be stabilized if its P content is partially or completely composed of CP. As an example, acidocalcisomes are amorphous and polyP-rich, at an acidic pH, with calcium and other cations, and polyP-metabolism-related enzymes; they are constrained within a membrane with a number of pumps, exchangers, and one identified channel (Docampo et al. 2010). As powerful anti-nucleation agents, linear polyPs were reported to be inhibitors of

dental calculus when applied topically, but "Pyrophosphate and linear polyphosphates, however, are easily hydrolyzed, chemically and enzymatically, whereas the phosphonates are very stable" (Briner and Francis 1973). This instability may be an advantage if temporary nucleation inhibition is desired.

12.6.2 Amorphous, P-Rich Phase Destabilization

Degens argued that kinetics controlled the spontaneous transformation of ACP to apatite (Degens 1976). However, spontaneous crystallization does not allow for biological control over this event. Fleisch and Neuman suggested that polyP could be an "ester phosphate fraction of serum … that is sufficiently large to contain one or several highly inhibitory polyphosphates, hydrolyzable by bone phosphatase" (Fleisch and Neuman 1960). Fleisch later noted that the addition of intestinal ALP to PPi-stabilized ACP removed the inhibition of the transformation from an amorphous to a crystalline state (Fleisch et al. 1968). They proposed that this might occur in biological mineralization processes. This relationship between pyrophosphate, pyrophosphatase, and ACP in biological calcification was also described by Wuthier et al. (1972).

The activity of alkaline phosphatase (ALP) (reviewed by Millán (2006)) in skeletal mineralization was first correlated with active skeletal mineral sites in 1923 by Robison (1923) and Martland and Robison (1924). Its inactivity in non-ossifying skeletal regions was identified soon after (Fell and Robison 1930). ALP was identified in fish (Lorch 1949c), the elasmobranch (shark, stingray, and family) skeleton (Lorch 1949b), and was later identified extracellular vesicles associated with calcium phosphate crystal formation in epiphyseal cartilage (Bonucci 1967). Lorch created a review of ALP being "an essential factor," with the caution that "the phosphatase mechanism alone cannot lead to full understanding of the complex processes of calcification and ossification" (Lorch 1949a). The demonstration that a calcium phosphate inhibitor in dog serum was destroyed by alkaline phosphatase and that the concentration of phosphoesters in blood was low enough to match the inhibition potential of polyP at the same low concentration (Fleisch and Neuman 1960) led to further bone mineral inhibition work. PolyP was later proven to be a bone mineral formation inhibitor; therefore, its destruction by a phosphatase enzyme was suggested as a mechanism for tissue mineralization (Fleisch and Neuman 1961).

When added to an incubating solution, PPi (identified in bone in 1956) (Cartier 1956) and polyP both inhibited calcium phosphate mineral formation in growing chick embryo femora in tissue culture at the mid- and high concentrations tested, but activated mineral formation at the lowest concentration (Fleisch et al. 1966). Fleisch et al. suggested that "partial destruction by a pyrophosphatase would then not only destroy this inhibition but might also lead to facilitation of calcium phosphate deposition" (Fleisch et al. 1966). Fleisch et al. commented that "ossifiable cartilage and bone contain enzymes which destroy polyphosphates" (Fleisch and Neuman 1961), but the citations that he referenced refer to pyrophosphatases. This highlights another complication in the literature and in the field of condensed

phosphates: terminology. Many terms such as metaphosphates, oligophosphates, and polyphosphates are used with different meanings. There are also many members of the phosphatase family that can cleave Pi from PPi or polyP in vivo, ex vivo, and in vitro. If ACP was stabilized by one of the CP species, phosphatase action during sample processing would destabilize ACP, which could accelerate the formation of calcium phosphate crystals.

12.7 Crystallization or Dissolution of Amorphous, P-Rich Solids

Phosphatases that cleave Pi from CP are not the only pathway for transformation of ACP into crystalline phases. Heat transforms ACP into different crystalline calcium phosphate phases (Lowenstam 1972); this technique has been used in microincineration studies (Policard 1929, 1942) of bacteria (Boyan-Salyers et al. 1978) and bone (Gay 1977). PPi detected in ash derived from granules generated by *Actinomyces* bacteria was thought to be condensed from high acid phosphate in the original material (Frazier and Fowler 1967). These granules were incinerated at a low temperature to decrease the organic content and then ignited at higher temperatures, which yielded ~ 40 % of P as PPi. Washed granules generated x-ray diffraction patterns of apatite that were weaker and more diffuse than normal bone. The infrared spectrum of low-temperature incinerated granules included apatite vibrations as well as other calcium phosphate species. The authors acknowledged the difficulty in identifying all of the components that could contribute to PPi formation. They did not specify the details of their PPi analysis; one of their citations for this method described an acid hydrolysis method that would quantify all CPs (Gee and Deitz 1955).

Gee and Deitz noted that acid phosphates can condense in a number of ways, so they used a careful analysis to demonstrate that synthetic dibasic calcium phosphates produce PPi with a manganous pyrophosphate separation procedure, but also commented that labile CP may form and be hydrolyzed in dilute acid (Gee and Deitz 1955). Additional IR absorption bands, in the regions of polyP and metaphosphates, were observed by ignition of octacalcium phosphate, but an assignment was not made (Fowler et al. 1966). This range of CP size and possibly hydrolysis reactions during sample processing or analysis procedures are some of the challenges with its identification. Pautard commented on the analysis of Ca- and P-rich granules produced by *Actinomyces bovis*, stating that the "heterogeneous nature of the granules, however, prohibits any firm conclusion as to the nature of the mineral within them" (Pautard 1970).

The electron beam can also affect CP stability. Bacterial polyP granules were observed to volatize under high electron density (reviewed by Harold (1966)). A reduction of energy required to volatize polyPs might add enough energy to an amorphous solid for it to crystallize. Docampo noted that the sample preparation method for electron microscopy affected the appearance of acidocalcisomes (Docampo et al. 2010). Electron-dense granules could become displaced during sectioning for TEM, "thus leaving holes in the sections" (Nilsson and Coleman

1977). Bonucci reviewed that fixation in salt solutions, decalcification during sectioning of ultrathin sections by water, and "mineral sublimation and reprecipitation due to the electron beam" are included on the list of technical difficulties in studying these solids by electron microscopy (Bonucci 1971).

Aqueous sample processing steps can dissolve polyP-containing solids. Ca-CP granules can exist as a discrete solid phase; volutin granules were thought to form by polyP precipitation within the high ionic strength cytoplasm (Harold 1963). Harold surmised that failed isolation of polyP-containing granules by differential centrifugation might have been caused by polyP dissolution in lower ionic strength media. The lower ionic strength media would solubilize the polyP, leaving it in the centrifugation supernatant. In his review of calcium phosphate microspheres in biology, Pautard reviewed how the uranyl acetate stain "dissolves the first-formed mineral complexes" (Pautard 1981). Baxter measured how the P "counts" by energy-dispersive spectroscopy of polyP granules in *Plectonema boryanum* decreased from freeze-dried from a liquid nitrogen slush, freeze-dried, air-dried to glutaraldehyde fixed (Baxter and Jensen 1980). Bonucci carefully noted that amorphous and crystalline calcifications within mitochondria have been identified and were dissolved by floating thin sections on 2 % formic acid (Bonucci et al. 1973). He built on previous suggestions (Thomas and Greenawalt 1968; Posner 1969) that this granular inorganic material might be a "subcrystalline precursor of calcium-deficient hydroxyapatite." The crystalline structures were von Kossa (phosphate) and alizarin red S (calcium) positive. No comment was made about the histological staining of the amorphous granules; the nature of these amorphous granules is therefore difficult to ascertain. Landis described how anhydrous preparation of mineralizing bone tissues revealed electron-dense mineral granules in bone cell mitochondria and clusters of small mineral particles in the extracellular space. This led him to conclude that bone mineralization occurs with the aggregation of discrete particulates (Landis et al. 1977b). As biological crystal precursors, their transient nature makes sense, if their components are to be readily mobilized and bioavailable.

The state of the cell has also been correlated to ACP stability. One theory involved the role of an "anti-calcifying factor." This factor, suggested to "protect healthy cells from mineralization, is the high-energy polyphosphates known to be present in large quantities within the intracellular compartment" (Fleisch 1964), citing (Follis 1960). Work by Henrichsen showed a correlation between cell death and calcification, and Fleisch wrote that this "might thus be explained by the disappearance of those condensed phosphates when the metabolism fails" (Fleisch 1964), referencing (Henrichsen 1958). Due to their inherent instability, detailed chemical analyses of biological, amorphous, Ca- and P-rich granules remain unclear.

12.8 CP Identification Within P-Rich Biological Solids

Particulate "ossific granules" were observed by Quekett, both inside and outside of cells (Quekett 1849), and tabulated by Watabe and Wilbur (1976). If these discrete "granules" are the building blocks of apatite biomineralization, the nature of the

precursors, which are commonly amorphous with respect to x-ray diffraction analysis, continues to be the subject of many studies. Lowenstam surveyed many organisms that mobilize phosphorus in amorphous, Ca- and P-rich granules (Lowenstam 1972). This chapter will review a few of these granules from different life forms.

The size, location, physical state (amorphous or crystalline), and chemical composition of these P-rich intracellular, intercellular (interpreted as extracellular, but within an extracellular matrix) "ossific" granules have been and continue to be the focus of much research effort. While some authors have commented on the possible roles of CPs in maintaining the amorphous nature of these crystalline precursors, direct identification of CP in these structures is a nontrivial problem. The effect of sample preparation on CPs has been noted, but not rigorously studied. Some techniques that are used to identify polyP in situ, such as red-shifted DAPI fluorescence, also label other species such as amorphous calcium phosphate and inositol phosphates (Omelon et al. 2016). Raman spectroscopy now offers a more specific in situ option for identification of the components in these solids (Kolozsvari et al. 2015). For example, the in vitro transformation of ACP to hydroxyapatite was successfully recorded with Raman spectroscopy (Kazanci et al. 2006). Although hydroxyapatite is not the mineral found in most biological phosphate minerals, it serves as a useful proxy and is commonly the name attributed to the product of biological phosphate mineralization processes, which Lowenstam classified in the 1980s.

12.9 Biomineralization Processes

Lowenstam differentiated two types of biomineralization processes, biologically induced and genetically controlled, and uses the term "mineral," even though minerals made by life forms do not meet the strict definition (Lowenstam 1972; Lowenstam and Weiner 1989). The biomineralization community's use of "mineral" – an identifiable crystalline structure with x-ray diffraction – will also be used in the rest of this chapter. With respect to the organism, biomineralization can be extracellular, intercellular, and/or intracellular. The mineral formation may not be genetically controlled in composition, size, or orientation within an organic matrix by some bacteria (prokaryotes) and algae (eukaryotes). The formation of minerals as a consequence of biological action could take place because the organism produced proteins to generate an extracellular organic matrix that changed the local chemical environment in a way that enabled crystal nucleation and growth. Extracellular matrices offer complex chemistry, with positive and/or negatively charged molecules that could affect the supersaturation required to precipitate minerals from the bulk environment. Biological actions that result in mineral formation could also begin in the intracellular environment and, by different processes, generate a locally increased ion concentration around the organism or within intracellular microenvironments that result in mineral precipitation within or near the cell (Weiner and Addadi 2011; Ferris et al. 1989).

While extracellular mineralization occurs in all five kingdoms, Lowenstam reviewed that intracellular biomineralization was defined to occur within

mitochondria, Golgi apparatus, or vesicles in a few bacteria and the eukaryotic kingdoms Protoctista, Animalia, and Plantae (Lowenstam 1981). Eukaryotes in particular are capable of producing an organized extracellular organic framework among which minerals of a specific chemistry, polymorph (crystal structure), size, and orientation (in shape and/or crystallographic axis) are distributed. These composite materials are commonly produced by eukaryotes to manufacture hard tissues such as shells and skeletons, minerals for storage for mineral metabolites (Lowenstam and Weiner 1983), or waste disposal sites (Simkiss 1980). This type of biomineral formation is assumed to occur through genetic control of many biological processes (Lowenstam 1981).

The fossil record suggests an increase in eukaryotes with tissues hardened by calcium carbonate, apatite, and quartz (silica) biomineralization in the late Proterozoic and early Cambrian. Synthesis of calcareous biominerals is widespread in eukaryotes, with only a few potential examples in prokaryotes (Lowenstam and Margulis 1980). This chapter will review examples of induced biological apatite formation through a CP pathway by a few prokaryotes and review possible relationships between eukaryotic, controlled, biological apatite formation and the chemical nature of intracellular, P-rich apatite precursors (noncrystalline, distinct phases that have the potential to form apatite by a chemical or thermal decomposition process) that may include CPs.

12.10 Biologically Induced Apatite Formation from Condensed Phosphates

12.10.1 Prokaryotes: Extracellular Mineralization

Watabe crafted a comprehensive chapter on "Calcium Phosphate Structures in Invertebrates and Protozoans" that summarizes the discovery of amorphous calcium phosphate solids within invertebrates and protozoans, noting that they are also identified in some eukaryotes. He summarized that "amorphous forms are more common than crystalline forms and appear to be protected against transformation to the crystalline state by some stabilizers within the tissues" (Watabe 1989). External to the life form, the stabilizing components may be destroyed and/or diffused from the granule, leaving an amorphous solid open to crystallization forces.

The potential for marine bacteria to concentrate P in a form that could create apatite was proposed by O'Brien et al., who discovered CFA deposits within bacteria embedded in nodules from the late Pleistocene to Holocene (O'Brien et al. 1981). They theorized that the CFA did not precipitate in vivo, but is most likely a "postmortem transformation of assimilated phosphorus," with the intracellular P sources being "nucleic acids, phospholipids, and polyphosphate." Extracellular biological apatite formation from intracellular polyP was proposed for the prokaryotes *Pseudomonas*, *Acinetobacter* (Nathan et al. 1993), *Thiomargarita* (Schulz and Schulz 2005), and *Beggiatoa* (Goldhammer et al. 2010), reviewed (Crosby and Bailey 2012), and recently demonstrated for marine symbionts in marine sponges

(Zhang et al. 2015). With *Beggiatoa*, Goldhammer et al. demonstrated a direct chemical link between intracellular polyP and apatite mineral (Goldhammer et al. 2010); therefore, it will be briefly and generally described.

These marine benthic prokaryotes uptake Pi from oxic seawater and concentrate intracellular Pi as polyP. When the surrounding dissolved oxygen concentration decreases, these organisms change their metabolic pathway and cleave the P-O-P bonds in the polyP stores to drive their biochemical processes. Pi created by P-O-P bond cleavage is secreted into the surrounding pore waters within the extracellular environment; Pi concentrations of up to 300 μM – much greater than the reported mean ocean bottom water (5 μM) – were measured (Schulz and Schulz 2005). Schulz and Schulz noted that Pi concentrations in seawater that are higher than 40 μM would precipitate hydroxyapatite, and this mineral was identified in the bacteria-rich sediment (Schulz and Schulz 2005). This process represents a biological pathway for biological phosphorus concentration – first as intracellular polyP and then as Pi in the extracellular environment that is constrained by the sediment and other organic matter and not diffused within the bulk seawater. Goldhammer et al. noted that apatite formation from intracellular polyP stores required live bacteria (Goldhammer et al. 2010), suggesting an active, extracellular apatite biomineralization process. Hirschler et al. reported apatite formation from aragonite ($CaCO_3$), ALP, and RNA as a P-source, without an active organism; the Pi concentration increase was attributed to the extracellular action of ALP (Hirschler et al. 1990).

12.10.2 Prokaryotes: Extracellular and Intracellular Mineralization

Another example of biologically induced calcium phosphate mineralization process by prokaryotes is that of oral bacteria. Intracellular phosphate calcification was identified in dental calculus in 1960 (Zander et al. 1960; Gonzales and Sognnaes 1960). The range of calcium phosphate minerals (apatite, whitlockite, and octacalcium phosphate) in the extracellular matrix that surrounds these bacteria (Legeros 1974) indicates an uncontrolled, biologically induced mineral process. Oral plaque phosphate mineral formation was determined not to correlate with calcium phosphate saturation state of saliva (Poff et al. 1997), yet these oral bacteria generate extracellular matrices with a calcium phosphate solid component. As is for the benthic bacteria, it may be the biologically driven concentration of calcium and phosphate within these local matrices that is more relevant to crystal nucleation.

Corynebacterium (*Bacterionema*) *matruchotii* is an oral bacterium that was studied for its ability to generate intracellular, Ca- and P-rich, amorphous solids when grown in a calcium-rich medium (Boyan-Salyers et al. 1978). These solids were analyzed at different time points after inoculation by washing and then ashing them at 700 °C. The first, ashed solid was not matched to a known mineral. After 6–10 days of culture, the ashed mineral was whitlockite, while older, ashed cultures presented apatite. The different crystalline compositions may have been related to the different ratios of total Ca, Mg, and P in the ashed granules. Ennever and

Creamer (1967) reviewed how amorphous calcium phosphate that was precipitated from a "calcifying solution" began to crystallize at 300 °C; therefore initially amorphous intracellular granules may have been calcified by the ashing process (Ennever and Creamer 1967). Older (6 week) *C. matruchotii* and cariogenic streptococcus cultures that were dried at temperatures at or less than 110 °C produced crystalline hydroxyapatite x-ray diffraction patterns (Moorer et al. 1993). They noted that calcification is a late event relative to inoculation, so that it is "possible that dying, metabolically incompetent, or dead cells trigger calcification phenomena." Ennever also induced *E. coli* to form intracellular calcium phosphate crystals, but only when grown in a metastable calcium phosphate medium and after the bacteria died (Ennever et al. 1974).

A noted shortcoming of *C. matruchotii* mineralization investigations was the hydroxyapatite supersaturation state in the calcifying media, which was close to or exceeded the value required for spontaneous hydroxyapatite nucleation (Ooi et al. 1981). Therefore, an analysis of the hydroxyapatite saturation state of the calcifying medium in which the *C. matruchotii* were grown was undertaken for Ca × P concentrations (measured in mg % Ca or P) ranging from below the value required for HAp mineral growth (35 $mg\%)^2$ and spontaneous HAp mineralization (50 $mg\%)^2$. The results indicated that intracellular calcium and phosphate deposits accumulated in the bacteria at saturation states as low as 35 mg^2. The conclusion that hydroxyapatite nucleation was enabled by the organism was supported by the observation of needlelike intracellular crystals, after sample processing embedding (Ooi et al. 1981).

A study of two strains of *C. matruchotii*, sectioned through unwashed bacterial aggregations, demonstrated intracellular and extracellular calcifications, depending on the strain and cell degradation. This study also identified electron-dense, amorphous material in the center of crystal clusters (Lie and Selvig 1974). Franker et al. subsequently isolated ALP activity in a strain of *C. matruchotii* (Franker et al. 1979), which opens up the possibility of ALP hydrolysis of CP associated with an intracellular amorphous granule. The amorphous granule, temporarily stabilized by a CP species, could initiate the formation of a crystalline solid after enzymatic CP hydrolysis.

Although published years earlier in Japan, Takazoe and Nakamura extracted and identified polyP in the metachromatic, amorphous, Ca- and P-rich intracellular granules of *C. matruchotii* (Takazoe and Nakamura 1965). Later electron microscopic and elemental analysis work demonstrated that *C. matruchotii* grown for 2 weeks or less, and sectioned while frozen and unfixed, contained amorphous granules with detectable Ca, Mg, and P compositions. Cultures grown for 3–4 weeks contained crystalline apatite, with detectable Ca and P elements. The authors concluded that there was probably a transition from an amorphous phase to an apatite phase within the bacterium.

More recent investigation of intracellular granules within *C. matruchotii* identified intracellular, Ca-containing granules approximately 0.5 μm in size with alizarin red and Ca-P-rich (with Mg and Si) 5 nm diameter granules with EDS that may have coalesced into larger (30–50 nm) granules (Linton et al. 2013). These granules

appeared to form chains that extruded from cells after 14 days of culture. At 21 days of growth, 0.25 μm-sized electron-dense granules with lucent cores were observed in the extracellular matrix. No measurement of electron-dense granule crystallinity was made, but evidence of organic "ghosts" was obtained with a chromium stain. This new work revealed a more direct link between the intracellular and extracellular, Ca- and P-rich granules generated and extruded by prokaryotes.

The prokaryotic members in oral bacteria are numerous. To investigate them as a group, Gonzales and Sognnaes imaged thin sections of undecalcified dental calculus fixed with osmium (Gonzales and Sognnaes 1960). They noted two populations of crystals that generated selected area electron diffraction patterns characteristic of apatite, brushite, and whitlockite. One population was similar in size to "bone" minerals (40 by 800 Å), and a second population was larger (500 by 26,000 Å). Intracellular crystallization was observed, but was associated with microorganism degeneration after encapsulation by a mineralized extracellular matrix, suggesting that live organisms contain material with mineralization potential, but the material remains amorphous when the organism was viable. In another study, examination by x-ray powder diffraction of dental calculus identified different minerals above and below the gingiva. Above the gingiva, octacalcium phosphate, whitlockite, and hydroxyapatite, with no brushite, were observed. Subgingival calculus was more crystalline and indicated whitlockite, with some evidence of hydroxyapatite, and different crystal morphology (Sundberg and Friskopp 1985). The different environments may have an effect on the crystalline phases; this is a hallmark of biologically induced mineralization. Gonzales and Sognnaes referenced the work of Pautard that identified intracellular apatite deposits in *Spirostomum ambiguum*, a calcifying eukaryotic protozoan (Pautard 1958).

12.11 Calcium Phosphate Solid Formation in Eukaryotes

Nilsson reviewed that lower organism biomineralization is considered to be an intracellular event, where the calcium deposits are formed within a membrane-bound space, while others summarize that higher organism calcification is extracellular (Nilsson and Coleman 1977). *Spirostomum ambiguum*, a eukaryotic protozoan that can produce intracellular crystalline calcium phosphate solids, is a notable exception. There is a wider literature of intracellular amorphous Ca- and P-rich granules, with increasing understanding of sample preparation effects on the appearance and/or crystallinity of these granules, the roles of mitochondria and phosphatases, stabilization of the amorphous phosphate state, and how CP stabilizes the amorphous state. Pautard summarized calcification events in unicellular organisms and gave the opinion that "it is not possible to do adequate justice to the seemingly endless nature and arrangement of inorganic deposits in unicellular animals and plants" and that the subject of mineral accumulation "cannot be a haphazard affair" (Pautard 1970). He speculated that there might be a connection between the processes by which unicellular organisms create intracellular solids and metazoan animals that orchestrate extracellular mineral formation.

Also informative is his perspective on the term "calcification," which has been used to describe calcium-rich clusters within an organelle or features within an organism. For example, an accumulation of calcium visible in an electron microscope could be called "calcified" should it be a dense crystalline solid or a calcium-rich region associated with organelles. This suggests a wide range of "calcifications" which may include a carbonate, phosphate or other anions, and a crystalline or amorphous state. Pautard's review included a 1894 publication that reported the formation of intracellular phosphate granules observed by light microscopy and chemical analysis (Schewiakoff 1894). Since then, the observed range in size, chemical composition, and physical state of intracellular P-rich granules continues to grow. Lowenstam (1972) and Watabe (1989) also wrote reviews that included possible links between prokaryote and eukaryote phosphate solid formation. Here, examples will be given of single-celled eukaryotes with amorphous, Ca- and P-rich, electron-dense granules that do not crystallize (*Tetrahymena*) and one that does (*Spirostomum ambiguum*), as well as higher eukaryotes with granules that do not crystallize (cestodes) and those that do: invertebrate inarticulate brachiopods and vertebrates. These organisms all share the privilege of living in close quarters with mitochondria.

12.11.1 Mitochondria as Phosphorus Concentrators

Mitochondria are intracellular organelles that produce ATP; they were also speculated to be involved in calcium phosphate biomineral (apatite) formation (Shapiro and Greenspan 1969) because of their ability to accumulate calcium and phosphate ions (Greenawalt et al. 1964). Mitochondria were associated with polyP in eukaryotes (Mudd et al. 1958; Lynn and Brown 1963), but it is possible that this information did not transfer to the biomineralization community at that time. It was theorized that mitochondria concentrated and then released the calcium and phosphate ions into the cytoplasm and then were transported into the extracellular matrix, where mineralization would occur (Shapiro and Greenspan 1969).

Weinbach and von Brand later extracted amorphous granules that were Ca- and P-rich, with "significant amounts" of carbonate and magnesium from rat liver mitochondria (Weinbach and von Brand 1967). Bonucci et al. probed the inorganic-organic relationship in intracellular mitochondrial calcifications from rat liver, myocardial, and skeletal muscle cells and a human carcinoma in great detail (Bonucci et al. 1973). They identified electron-dense granular and crystalline materials and noted the co-location of organic material with these deposits. Microincineration of rat liver mitochondria that were incubated in conditions to accumulate calcium and phosphate detected whitlockite particles (Thomas and Greenawalt 1968). Gay and Schraer observed that electron-dense granules (EDG) in mitochondria of osteoblasts prepared as frozen thin sections were sensitive to sample preparation (Gay and Schraer 1975). Jones reviewed that the proposal that mitochondria are involved in mineralization does not comment on the transport of the mitochondrial EDG to the surrounding tissue (Jones 1969). With modern

cryo-sample preparation methods, mitochondria were observed interacting with Ca- and P-rich vesicles that are electron dense in murine osteoblast (bone-forming) cell cultures (Boonrungsiman et al. 2013).

On the opposite site of the temperature scale, incinerated dense mitochondrial granules isolated from rat liver mitochondria generated apatite and whitlockite x-ray diffraction patterns (Weinbach and Vonbrand 1965) as well as MgO "presumably derived from $MgCO_3$" (Weinbach and von Brand 1967) as the major inorganic constituents. The authors concluded that these amorphous granules "formed under these in vitro conditions is similar in many respects to that of the calcareous corpuscles formed in vivo by cestodes and to the intracellular deposition of hydroxyapatite in the ciliate *Spirostomum ambiguum*" (Weinbach and von Brand 1967).

12.11.2 Intracellular, Ca- and P-Rich Solids

12.11.2.1 *Tetrahymena pyroformis* (Protozoan)

The ciliated protozoa *Tetrahymena geleii* was investigated for its phosphatase activity in 1951, when it was determined that it displayed intracellular acid and alkaline phosphatase activity (Elliott and Hunter 1951). It was later reported that aging mitochondria in *Tetrahymena pyroformis* generated dense granules (Elliott and Bak 1964). Subsequently, intracellular, refractile, 0.5–3 um diameter granules in *T. pyroformis* were that stained positively with lead (Rosenberg 1966). This lead-positive stain suggested a polyP content, as reported for other metachromatic intracellular granules by Ebel et al. (1958a, b). Further tests confirmed that the granules contained CP, because they did not dissolve in water, but would dissolve in EDTA solutions at pH 7.0, by contact with sodium ion exchange resins or by acidification. The granules were discovered to contain mostly PPi, with traces of higher polymers, by chromatography. This lead to the conclusion that the granules were "hydrated, mixed salt of calcium magnesium pyrophosphate" that "gave no indication of the presence of microcrystalline material" when examined with x-ray diffraction (Rosenberg 1966).

Electron microprobe analysis of the granules revealed calcium, magnesium, and phosphorus (Coleman et al. 1972) in an "apatite-like material" (reviewed in Nilsson and Coleman 1977) that was similar to granules found in the *T. pyroformis* mitochondria. These amorphous "membrane-bound, electron-dense granules were thought to resemble the granules involved in biomineralization in invertebrates" (Nilsson and Coleman 1977; citing (Simkiss 1976)).

12.11.2.2 Cestodes (Non-chordate Metazoan)

Amorphous "calcareous corpuscles," ranging in sizes from 2 to 34 μm, which may be calcified by smaller granules < 1 μm in size (Chowdhury and Derycke 1977) and composing up to 41 % of the organism's dry weight (Diamare 1930) (cited in Nieland and von Brand (1969)) were identified in different species of cestodes (tapeworm) (Pautard 1966). The granules stained positively with "von Kossa's silver test for calcium" (Chowdhury et al. 1955) (von Kossa is a stain for phosphate

(Meloan and Puchtler 1985)) and nuclear fast red (for calcium, referencing (McGee-Russell 1955)). Von Brand et al. reported that "Some appeared heavily calcified and are uniformly black; others are almost exclusively stained in the center, and every kind of intermediary stage can be found" (von Brand et al. 1960). A later description of an elemental composition was "phosphorus" instead of phosphate (von Brand et al. 1967). A positive reaction for alkaline phosphatase with Gomori's modified technique was reported for the corpuscles in *Taenia saginata* (Chowdhury et al. 1955). Von Brand et al. described a corpuscle isolation process that involved dissolution in 5 % aqueous KOH and 1 h heating on a boiling water bath; the possible effect of this sample treatment on CP degradation could be considered. Granules extracted this way diffracted hydroxyapatite and brucite (von Brand et al. 1960).

The corpuscles are first formed in the nuclei; calcifications are later observed in vacuoles as concentric layers or whorls and can eventually fill and outgrow the cell (reviewed in Chowdhury and Derycke 1977). Phospholipids were theorized to "be associated with the movement of Ca++ and other ions from the environment as well as their precipitation" (Chowdhury and Derycke 1976). These roles could also be associated with the calcium sequestration and crystal inhibition properties of CP, as may also occur within mitochondria. Extracted corpuscles from a range cestode species were amorphous and consisted primarily of Ca, Mg, P, and CO_2 (Scott et al. 1962). The amorphous solids were stable at 150 °C for 122 days, but heating at 180 °C for 7 days nucleated dolomite (Ca, $MgCO_3$) mineral, while heating at 400 °C for 4 h also generated magnesium oxide, and 18 days also generated hydroxyapatite (von Brand et al. 1969). The amorphous calcium phosphate phase was presumed to be stabilized by the high magnesium and carbonate content in the corpuscles. The technique used to measure the P content involved isolating, drying, weighing, dissolving the corpuscles in 3N HCl, and measuring the Pi with colorimetry; this technique may accelerate CP hydrolysis so CP may not be detected (von Brand et al. 1965). CP species were not discussed as possible components in these amorphous, intracellular cestode granules.

12.11.2.3 *Spirostomum ambiguum* (Protozoan)

With x-ray diffraction, Pautard identified hydroxyapatite particles within dried whole bodies of this unicellular eukaryote (Pautard 1958). A good match was made with ox bone mineral, leading him to conclude that the organism contained apatite minerals. Soon after, intracellular phosphatase activity within *S. ambiguum* was identified (Padmavathi 1958). X-ray diffraction analysis of younger animals generated a more diffuse x-ray diffraction pattern with unidentified diffraction rings (Pautard 1959). Older animals were crushed in ice and separated by centrifugation. A mid-density fraction of 0.5–3 μm dense granules generated hydroxyapatite diffraction patterns. These granules were composed of strings of 65 × 215 Å-sized, cigar-shaped particles. The oldest animals diffracted with the strongest hydroxyapatite reflections, and the larger (up to 1 μm) solids were in the shape of plates. The smaller crystallite size calculated from the diffraction patterns suggests the plates were agglomerations, and this was confirmed by observing smaller plates that seemed to grow with the addition of smaller particles. Pautard proposed that the

cigar-shaped particles had a "resemblance to the generally accepted size for the primary particles in metazoan bone" (Pautard 1959).

Apatite in the endoplasm was suggested to be waste products from "mitochondrial accumulations" (Jones 1967), as "many mitochondria" were associated with the vacuoles in which the first intracellular granules were located (Pautard 1970). Electron microprobe investigations correlated calcium-rich areas with mitochondria and/or vesicles and bundles of microfilament (Osborn and Hamilton 1977). It was theorized that these intracellular apatite granules could provide stiffness to improve the burrowing skills of older animals (Bien and Preston 1968). Reports on mineral formation within *S. ambiguum* did not discuss or attempt to identify or extract CPs from the organism.

Pautard pointed out that for most studies of phosphate-calcified organisms, "the evidence for any crystal structure has been gathered from dead and dried specimens, but whereas in a cell free, relatively permanent product such as vertebrate tooth enamel such well-crystallized material can be examined with little chance of post mortem change…" (Pautard 1981). He proposed that many vertebrate and invertebrate organisms could biomineralize with "structures" formed inside the cell and "either retained or expelled to the immediate environment" (Pautard 1981), which leads to the discussion of controlled mineralization of eukaryotic extracellular matrices.

12.12 Extracellular, Ca- and P-Rich, Crystalline Solids

The processes of extracellular phosphate mineral formation in higher eukaryotes are challenging to characterize, as they require sample preparation and sectioning which can dissolve or change the nature of unstable solid phases. The identification of CPs is also less probable after extensive sample preparation, especially if the sample contains phosphatases that could accelerate their degradation. Even with sample preparation challenges, Lowenstam identified noncrystalline calcium-phosphorus deposits (sometimes with magnesium) in some mature tooth denticles of *Polyplacophora* (mollusca), a polychete, gizzard plates of some gastropods, the gill support of a bivalve, the carapace of an arthropoda that transformed into dahllite (and sometimes also calcite), or whitlockite (Lowenstam 1972). Brown's extensive review of metal-containing "granules" in invertebrate tissues includes a summary of the variations in Ca- and P-rich granules and suggests four granule initiation sites: the Golgi body or its associated cisternae, the endoplasmic reticulum, mitochondria, and/or an unknown origin (Brown 1982), suggesting association between the granules and cellular transport mechanisms.

A review of all of the Ca-P amorphous solids in higher eukaryotes will not be undertaken. Instead, a focused review of the intracellular, amorphous Ca- and P-rich solids that are putative precursors for extracellular, crystalline apatite formation in the shell of some inarticulate brachiopods, and the vertebrate skeleton, with the perspective of the potential role(s) for CPs in the extracellular apatite crystallization process is presented.

12.12.1 Invertebrate Atremate Brachiopod Shells

Iwata published a detailed review on this bivalve, phosphatic shell and confirmed McConnell's identification of francolite (a CFA with a higher F content than dahllite) in the shell of the inarticulate brachiopod *Lingula* (Iwata 1981; McConnell 1973). Legeros et al. expanded this identification to *Glottidia pyramidata*, as well as *Lingula adamsi* and *L. anatine* (Legeros et al. 1985). Ultrastructure work of the *L. unguis* shell described how the first layer that lies underneath the cells that produce the shell contained amorphous granules 10–50 nm in diameter. The layers further below contained larger (~35 × 125 × 150 nm) acicular crystals, with the largest crystals at the shell surface. No intracellular mineralization was noted, but the aggregation of "amorphous crystal nuclei" was thought to grow into the larger crystals (Iwata 1981).

The shell of another brachiopod (*G. pyramidata*) was also noted to include a layer of aggregated, apatitic spherulites in the shell and the presence of vacuoles containing electron-dense granules of apatite and ACP. It was proposed that the granules may have derived from rough endoplasmic reticulum and transported to extracellular spaces (Watabe and Pan 1984; Pan and Watabe 1988a). Pan noted different squamous cells in the mantle epithelium that generate the shell; one cell type contained granules within large vacuoles that were also observed in the extrapallial space. One other cell type also had abundant mitochondria, as well as granule-containing vacuoles (Pan and Watabe 1988a). "Columnar cells" contained intracellular electron-dense, Ca-, P-, and S-rich granules that were approximately 0.1–0.5 μm in diameter. Their exact amorphous or crystalline nature was not established, as they disappeared by uranyl acetate staining or by a strong electron beam or gave x-ray diffraction patterns of a range of apatite minerals (Watabe and Pan 1984). This instability was attributed with their use for shell apatite formation.

The shell of the brachiopod *Discina* was described in a detailed report to be composed of coated, subrounded granules of apatite, approximately 4–8 nm in diameter coated with an organic matrix (Williams et al. 1992). Aggregation of these granules was observed in intracellular vesicles and intercellular spaces within interdigitating tubes; at ends of tubes, further granule aggregation occured. Their final aggregation products were larger spheres or "relatively smooth surfaces over several square micrometers, sporadically studded with low domes up to 100 nm in diameter," blades, or platelets. The mineral components of the shell were apatite spheres coated with a medium electron-dense coat that did not dissolve in a decalcification process and were less than 10 n in size. The crystallinity of the intracellular or extracellular spheres was not evaluated; it was assumed that the exocytosed granules were mostly crystalline as they retained a spherical shape, "with comparatively few signs of post-deformational compression." Assessments of potential CP content of these granules were not made.

The extraction of proteins from the *L. anatina* shell was shown to destabilize in vitro ACP particles and produced in vitro carbonate-substituted fluorapatite from buffered metastable solutions containing calcium, phosphate, and fluoride (Leveque et al. 2004). This experiment could not identify the potential phosphatase effect of

cleaving CPs that, when intact, could stabilize ACP. This biochemical and the previous ultrastructural studies did not examine the potential roles of CPs or ALP in C(F)A biomineralization of the shell. The factors that affect the transition the x-ray amorphous to crystalline states within the extracellular matrices of the inarticulate brachiopod are still unknown.

Radioactive tracer work indicated that shell calcium originates directly from seawater; however, Pi from seawater did not follow a similar direct pathway. The authors suggested that the major Pi source for the shell mineral was food rather than seawater (Pan and Watabe 1988b). The use of digested Pi for shell mineralization suggests a more biochemically controlled and still undefined biochemical pathway for phosphate biomineralization. However, the study of atremate shell biomineralization pathways has identified the agglomeration of amorphous, granular, Ca- and P-rich solids.

12.12.2 Vertebrates (Metazoans)

The skeletal mineralization literature is extensive, and the horizon of understanding of the inorganic chemistry involved in skeletal biological apatite formation remains largely ahead of us. This section will review the smaller set of CP literature on the topic of skeletal mineralization.

After the observation of ossific granules by Quekett (1849), the dynamic and transient nature of inorganic skeletal granules was reported decades ago. Fitton reported very dense particles, less than 100 Å in diameter between collagen fibers in undecalcified, embryonic avian bone (Fitton Jackson 1957). These discrete granules showed no preferred orientation with respect to the collagen and suggested a tissue mineralization process initiated by discrete particles that either were CFA or transformed to apatite during sample preparation. There is an extensive literature that identifies amorphous, Ca- and P-rich granules in the mitochondria and extracellular matrix of mineralizing skeleton if sample preparation is anhydrous (Landis et al. 1977b) or cryo-techniques are used (Molnar 1959; Landis et al. 1977a; Mahamid et al. 2011). These efforts have identified electron-dense, amorphous solids with a Ca:P ratio less than one when the granule is closer to the cell, with an increasing value when the granules are located further from the cell in the extracellular matrix. Brown and Chow reviewed that the supposed ACP component of bone mineral did not hydrolyze as rapidly as synthetic ACP; this and other factors suggested that the content of bone mineral that can be interpreted as amorphous is not similar to synthetic ACP (Brown and Chow 1976). However, a small addition of CP to ACP would be expected to increase its stability dramatically.

Pautard proposed that microspheres "seemed to be assembled and mineralized in the Golgi apparatus of the osteocyte (bone cell), later to be dispatched to the extracellular space and arranged in a characteristic pattern," but the nature of the P-components in the granules was not known (Pautard 1981). Aaron et al. extracted and characterized 1 μm calcified microspheres from the bone, which included a "Complex substructure of clusters of non-collagenous calcified filaments

surrounding a less dense centre" (Aaron et al. 1999). Degens reviewed that 100 nm-sized vesicles with inorganic pyrophosphatase, ATPase, and alkaline phosphatase are located in intercellular matrices of calcified tissues. He postulated that the pyrophosphatase would hydrolyze the PPi into Pi, enabling phosphate mineral crystallization (Degens 1976). Bisaz et al. isolated PPi from bovine and human teeth (Bisaz et al. 1968). Anderson-Reynolds concluded that "mineralization might be triggered by the enzymatic hydrolysis of PPi" (Anderson and Reynolds 1973).

A combination of the theory that a mineralization-promoting phosphatase destroyed a mineralization inhibitor, which is reviewed in Sects. 12.6.1 and 12.6.2, with the theory that a phosphatase "boosts" the local Pi concentration, increasing calcium phosphate mineral saturation, was suggested in 1966 (Fleisch et al. 1966). The possible dual role of CPs acting as crystallization inhibitors and, when destroyed, phosphate mineral promoters was more recently reviewed (Omelon and Grynpas 2008; Omelon et al. 2013). However, much of the evidence for the role of CP as a skeletal apatite precursor was circumstantial, until ex vivo identification of polyP was made at the tissue scale, at the apatite mineralization front in elasmobranch (shark, stingray, and relative) skeletons with Raman spectroscopy. Frozen samples were cryo-sectioned and immediately examined, as it was theorized that sectioning released endogenous ALP onto the section surface where it could accelerate CP degradation. Exogenous ALP application demonstrated a reduction in DAPI and toluidine blue staining, signaling a reduction in negative polyanion concentration. The formation of Pi in the same mineralization front locations where CP was transiently stained was indicated with von Kossa staining (Omelon et al. 2014).

Analytical limitations for observing in vivo mineralization process have been reimagined with the recent and simultaneous use of fluorescent probes and Raman spectroscopy to identify ACP at the mineral formation front in the zebrafish skeleton (Bennet et al. 2014), as well as cryo-SEM (Akiva et al. 2015). Searching in different locations for calcium phosphate mineral precursors, such as blood vessel lumen of rapidly mineralizing chicken embryo, is also fruitful (Kerschnitzki et al. 2016). New analytical techniques that minimized tissue disruption and identify specific molecules have opened promising windows into the microscopic, biological, amorphous, and crystalline calcium phosphate solid formation processes in extracellular mineralizing tissues, which still hold many chemical and biochemical secrets about phosphate mineralization control.

Conclusions

Investigation into role(s) of CPs in biological calcium phosphate solid formation is only decades old. CP identification in concentrated Ca- and P-rich, amorphous granules in *C matruchotii* and *Tetrahymena* occurred in the 1960s. The pathway of P concentration and apatite formation in pore waters by some marine benthic bacteria and the formation of skeletal apatite in elasmobranch skeletal mineral are more recently reported. This chapter outlines how CP chemistry can assist with concentrating calcium and phosphorus, without exceeding the saturation to induce calcium phosphate mineral nucleation. It also reviews how CPs inhibit mineral nucleation and stabilize amorphous calcium phosphate phases until

these properties are removed when CPs are broken down by spontaneous hydrolytic degradation or phosphatase enzymes.

With only suggestions of relationships between the CP-ALP-ACP triad in prokaryotic phosphate mineral formation and some in vitro arguments for their relevance, the evidence that these three species are involved in controlling the phosphate mineral chemistry of eukaryotic, extracellular skeletal mineralization is still scarce. The eukaryotic cell nondisclosure agreement remains intact; their micron-level engineering plans and processes are still largely unidentified. Their secrets are largely hidden within the small, constrained, and dynamic microenvironments that remain outside the limits of our most common sample preparation and analytical tools. However, current and creative analytical developments will create the opportunity for more discoveries of the presence or absence of CPs in controlled, biological, calcium phosphate amorphous, and crystalline solid phase formation.

Acknowledgments Sidney Omelon would like to acknowledge funding from the Volkswagen Foundation Experiment! Initiative, the Natural Sciences and Engineering Research Council of Canada Discovery Grant program, and the librarians at the Max Planck Institute for Colloids and Interfaces for locating chapter resource materials.

References

Aaron JE, Oliver B, Clarke N et al (1999) Calcified microspheres as biological entities and their isolation from bone. Histochem J 31(7):455–470. doi:10.1023/a:1003707909842

Akiva A, Malkinson G, Masic A et al (2015) On the pathway of mineral deposition in larval zebrafish caudal fin bone. Bone 75:192–200. doi:10.1016/j.bone.2015.02.020

Amjad Z (1998) Calcium sulphate dihydrate (gypsum) scale formation on heat exchanger surfaces: the influence of scale inhibitors. J Coll Interf Sci 123(2):523–536

Anderson HC, Reynolds JJ (1973) Pyrophosphate stimulation of calcium uptake into cultured embryonic bones – fine-structure of matrix vesicles and their role in calcification. Dev Biol 34(2):211–227. doi:10.1016/0012-1606(73)90351-5

Baxter M, Jensen T (1980) A study of methods for in situ X-ray-energy dispersive analysis of polyphosphate bodies in *Plectonema-boryanum*. Arch Microbiol 126(3):213–215. doi:10.1007/Bf00409922

Bennet M, Akiva A, Faivre D et al (2014) Simultaneous raman microspectroscopy and fluorescence imaging of bone mineralization in living zebrafish larvae. Biophys J 106(4):L17–L19. doi:10.1016/j.bpj.2014.01.002

Betts F, Blumenthal NC, Posner AS (1981) Bone mineralization. J Cryst Growth 53(1):63–73. doi:10.1016/0022-0248(81)90056-7

Bien SM, Preston FB (1968) Calcification of *Spirostomum ambiguum*. J Protozool 15(2):251–256. doi:10.1111/j.1550-7408.1968.tb02117.x

Bisaz S, Russell RGG, Fleisch H (1968) Isolation of inorganic pyrophosphate from bovine and human teeth. Arch Oral Biol 13(6):683–696. doi:10.1016/0003-9969(68)90146-5

Bonucci E (1967) Fine structure of early cartilage calcification. J Ultra Mol Struct R 20(1–2):33–50. doi:10.1016/S0022-5320(67)80034-0

Bonucci E (1971) Locus of initial calcification in cartilage and bone. Clin Orthop Relat R 78:108–139

Bonucci E, Derenzin M, Marinozz V (1973) Organic-inorganic relationship in calcified mitochondria. J Cell Biol 59(1):185–211. doi:10.1083/jcb.59.1.185

Boonrungsiman S, Fearn S, Gentleman E et al (2013) Correlative spectroscopy of silicates in mineralised nodules formed from osteoblasts. Nanoscale 5(16):7544–7551. doi:10.1039/c3nr02470a

Boyan-Salyers BD, Vogel JJ, Ennever J (1978) Pre-apatitic mineral deposition *in Bacterionema-matruchotii*. J Dent Res 57(2):291–295. doi:10.1177/00220345780570022501

Briner WW, Francis MD (1973) In-vitro and in-vivo evaluation of anti-calculus agents. Calc Tiss Res 11(1):10–22. doi:10.1007/Bf02546592

Brown WE, Chow LC (1976) Chemical properties of bone-mineral. Annu Rev Mater Sci 6:213–236. doi:10.1146/annurev.ms.06.080176.001241

Brown BE (1982) The form and function of metal-containing 'granules' in invertebrate tissues. Biol Rev 57(4):621–667 doi:10.1111/j.1469-185X.1982.tb00375.x

Cartier P (1956) Separation and identification of pyrophosphates in bony tissue. C R Acad Sci 243:982–985

Chowdhury N, Derycke PH (1976) Qualitative distribution of neutral lipids and phospholipids in *Hymenolepis microstoma* from Cysticercoid to egg producing adult. Z Parasitenkd 50(2): 151–160. doi:10.1007/Bf00380519

Chowdhury N, Derycke PH (1977) Structure, formation, and functions of calcareous corpuscles in *Hymenolepis microstoma*. Z Parasitenkd 53(2):159–169. doi:10.1007/Bf00380461

Chowdhury AB, Dasgupta B, Ray HN (1955) Kernechtrot or nuclear fast red in the histochemical detection of calcareous corpuscles in *Taenia saginata*. Nature 176(4484):701–702

Coleman JR, Nilsson JR, Warner RR et al (1972) Qualitative and quantitative electron-probe analysis of cytoplasmic granules in *Tetrahymena pyriformis*. Exp Cell Res 74(1):207–219. doi:10.1016/0014-4827(72)90499-5

Crosby CH, Bailey JV (2012) The role of microbes in the formation of modern and ancient phosphatic mineral deposits. Front Microbiol 3:1–7, ARTN 241 10.3389/fmicb.2012.00241

Darton EG (1997) Scale inhibition techniques used in membrane systems. Desalination 113 (2–3):227–229. doi:10.1016/S0011-9164(97)00133-1

de Jager HJ, Heyns AM (1998) Kinetics of acid-catalyzed hydrolysis of a polyphosphate in water. J Phys Chem A 102(17):2838–2841. doi:10.1021/jp9730252

Degens ET (1976) Molecular mechanisms on carbonate, phosphate, and silica deposition in the living cell, vol 64. Topics in current chemistry. Springer, Berlin

Diamare V (1930) Note d'istofisiologia sui cestode II. Sui corpuscoli calcarei dei cestode Rinascenza Medica 7:315–316

Dick RP, Tabatabai MA (1986) Hydrolysis of polyphosphates in soils. Soil Sci 142(3):132–140. doi:10.1097/00010694-198609000-00002

Docampo R, Ulrich P, Moreno SNJ (2010) Evolution of acidocalcisomes and their role in polyphosphate storage and osmoregulation in eukaryotic microbes. Philos T R Soc B 365(1541): 775–784. doi:10.1098/rstb.2009.0179

Ebel JP, Colas J, Muller S (1958a) Recherches cytochimiques sur les polyphosphates inorganiques contenus dans les organismes vivants II Mise au point de méthodes de détection cytochimiques spécifiques des polyphosphates. Exp Cell Res 15(1):28–36, http://dx.doi.org/10.1016/0014-4827(58)90059-4

Ebel JP, Colas J, Muller S (1958b) Recherches cytochimiques sur les polyphosphates inorganiques contenus dans les organismes vivants III Présences de polyphosphates chez divers organisms inférieurs. Exp Cell Res 15(1):36–42, http://dx.doi.org/10.1016/0014-4827(58)90060-0

Elliott AM, Bak IJ (1964) Fate of mitochondria during aging in *Tetrahymena pyriformis*. J Cell Biol 20(1):113–129. doi:10.1083/jcb.20.1.113

Elliott AM, Hunter RL (1951) Phosphatase activity in Tetrahymena. Biol Bull 100(3):165–172. doi:10.2307/1538526

Ennever J, Creamer H (1967) Microbiologic calcification: bone mineral and bacteria. Calc Tiss Res 1(1):87–93. doi:10.1007/bf02008078

Ennever J, Vogel JJ, Streckfu J (1974) Calcification by *Escherichia coli*. J Bacteriol 119(3):1061–1062

Fell HB, Robison R (1930) The development and phosphatase activity in vivo and in vitro of the mandibular skeletal tissue of the embryonic fowl. Biochem J 24(6):1905–1921
Ferris FG, Tazaki K, Fyfe WS (1989) Iron-oxides in acid-mine drainage environments and their association with bacteria. Chem Geol 74(3–4):321–330. doi:10.1016/0009-2541(89)90041-7
Fitton Jackson S (1957) The fine structure of developing bone in the embryonic fowl. Proc Roy Soc Lond Ser B Biol Sci 146(923):270–280
Fleisch H (1964) Role of nucleation and inhibition in calcification. Clin Orthop Relat R 32:170–182
Fleisch H, Bisaz S (1962) Isolation from urine of pyrophosphate, a calcification inhibitor. Am J Physiol 203(4):671
Fleisch H, Neuman WF (1960) On the role of phosphatase in the nucleation of calcium phosphate by collagen. J Am Chem Soc 82(14):3783–3784. doi:10.1021/ja01499a076
Fleisch H, Neuman WF (1961) Mechanisms of calcification – role of collagen, polyphosphates, and phosphatase. Am J Physiol 200(6):1296–1300
Fleisch H, Strauman F, Schenk R et al (1966) Effect of condensed phosphates on calcification of chick embryo femurs in tissue culture. Am J Physiol 211(3):821–825
Fleisch H, Russell RGG, Bisaz S et al (1968) Influence of pyrophosphate on transformation of amorphous to crystalline calcium phosphate. Calc Tiss Res 2(1):49–59. doi:10.1007/Bf02279193
Follis RH Jr (1960) Calcification of cartilage. In: Sognnaes RF (ed) Calcification in biological systems: a symposium presented at the Washington meeting of the American association for the advancement of science, December 29, 1958. American Association for the Advancement of Science, Bailey, p 511
Fowler BO, Moreno EC, Brown WE (1966) Infra-red spectra of hydroxyapatite octacalcium phosphate and pyrolysed octacalcium phosphate. Arch Oral Biol 11(5):477–492. doi:10.1016/0003-9969(66)90154-3
Francis MD (1969) Inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates. Calc Tiss Res 3(2):151–162. doi:10.1007/Bf02058658
Franker CK, McGee MP, Rezzo TP (1979) Alkaline-phosphatase activity in a strain of *Bacterionema matruchotii*. J Dent Res 58(7):1705–1708. doi:10.1177/00220345790580071001
Frazier PD, Fowler BO (1967) X-ray diffraction and infrared study of sulphur granules of *Actinomyces bovis*. J Gen Microbiol 46:445–450
Gadaleta SJ, Paschalis EP, Betts F et al (1996) Fourier transform infrared spectroscopy of the solution-mediated conversion of amorphous calcium phosphate to hydroxyapatite: new correlations between x-ray diffraction and infrared data. Calcif Tiss Int 58(1):9–16. doi:10.1007/Bf02509540
Gal A, Weiner S, Addadi L (2015) A perspective on underlying crystal growth mechanisms in biomineralization: solution mediated growth versus nanosphere particle accretion. CrystEngComm 17(13):2606–2615. doi:10.1039/c4ce01474j
Gay CV (1977) Ultrastructure of extracellular phase of bone as observed in frozen thin-sections. Calc Tiss Res 23(3):215–223. doi:10.1007/Bf02012788
Gay C, Schraer H (1975) Frozen thin-sections of rapidly forming bone – bone cell ultrastructure. Calc Tiss Res 19(1):39–49. doi:10.1007/Bf02563989
Gee A, Deitz VR (1955) Pyrophosphate formation upon ignition of precipitated basic calcium phosphates. J Am Chem Soc 77(11):2961–2965. doi:10.1021/ja01616a009
Gilmore BH (1937) Prevention of calcium deposits in process waters – relative value of sodium metaphosphate and pyrophosphate. Ind Eng Chem 29:584–590. doi:10.1021/ie50329a024
Goldhammer T, Bruchert V, Ferdelman TG et al (2010) Microbial sequestration of phosphorus in anoxic upwelling sediments. Nat Geosci 3(8):557–561. doi:10.1038/ngeo913
Gonzales F, Sognnaes RF (1960) Electron microscopy of dental calculus. Science 131(3394):156–158. doi:10.1126/science.131.3394.156
Greenawalt JW, Rossi CS, Lehninger AL (1964) Effect of active accumulation of calcium + phosphate ions on structure of rat liver mitochondria. J Cell Biol 23(1):21–38. doi:10.1083/jcb.23.1.21

Habraken WJEM, Tao JH, Brylka LJ et al (2013) Ion-association complexes unite classical and non-classical theories for the biomimetic nucleation of calcium phosphate. Nat Commun 4. doi:ARTN 150710.1038/ncomms2490

Harold FM (1963) Inorganic polyphosphate of high molecular weight from *Aerobacter aerogenes*. J Bacteriol 86(4):885–887

Harold FM (1966) Inorganic polyphosphates in biology – structure, metabolism and function. Bacteriol Rev 30(4):772–794

Hatch GB, Rice O (1939) Surface-active properties of hexametaphosphate. Ind Eng Chem 31: 51–57. doi:10.1021/ie50349a012

Hatch GB, Rice O (1945) Threshold treatment of water systems – corrosion control and scale prevention with glassy phosphate. Ind Eng Chem 37(8):710–715. doi:10.1021/ie50428a013

Henrichsen E (1958) Alkaline phosphatase and calcification: histochemical investigations on the relationship between alkaline phosphatase and calcification. Acta Orth Scand 29(Suppl 34):5–85

Hirschler A, Lucas J, Hubert JC (1990) Bacterial involvement in apatite genesis. FEMS Microbiol Ecol 73(3):211–220. doi:10.1016/0378-1097(90)90732-6

Iwata K (1981) Ultrastructure and mineralization of the shell of *Lingula unguis* Linne, (Inarticualte Brachiopod). J Fac Sci Hokkaido Univ Ser IV 20(1):35–65

Jones AR (1967) Calcium and phosphorus accumulation in *Spirostomum ambiguum*. J Protozool 14(2):220–225. doi:10.1111/j.1550-7408.1967.tb01987.x

Jones AR (1969) Mitochondria, calcification and waste disposal. Calc Tiss Res 3(4):363–354. doi:10.1007/Bf02058679

Karl-Kroupa E, Callis CF, Seifter E (1957) Stability of condensed phosphates in very dilute solutions. Ind Eng Chem 49(12):2061–2062. doi:10.1021/ie50576a049

Kazanci M, Fratzl P, Klaushofer K et al (2006) Complementary information on in vitro conversion of amorphous (precursor) calcium phosphate to hydroxyapatite from Raman microspectroscopy and wide-angle X-ray scattering. Calcif Tissue Int 79(5):354–359. doi:10.1007/s00223-006-0011-9

Kerschnitzki M, Akiva A, Ben Shoham A et al (2016) Transport of membrane-bound mineral particles in blood vessels during chicken embryonic bone development. Bone 83:65–72. doi:10.1016/j.bone.2015.10.009

Ketrane R, Saidani B, Gil O et al (2009) Efficiency of five scale inhibitors on calcium carbonate precipitation from hard water: effect of temperature and concentration. Desalination 249(3):1397–1404. doi:10.1016/j.desal.2009.06.013

Kolozsvari B, Firth S, Saiardi A (2015) Raman spectroscopy detection of phytic acid in plant seeds reveals the absence of inorganic polyphosphate. Mol Plant 8(5):826–828. doi:10.1016/j.molp.2015.01.015

Kulaev IS, Vagabov VM, Kulakovskaya TV (2004) The biochemistry of inorganic polyphosphates, 2nd edn. Wiley, Chichester

Landis WJ, Hauschka BT, Rogerson CA et al (1977a) Electron-microscopic observations of bone tissue prepared by ultra-cryomicrotomy. J Ultra Mol Struct R 59(2):185–206. doi:10.1016/S0022-5320(77)80079-8

Landis WJ, Paine MC, Glimcher MJ (1977b) Electron-microscopic observations of bone tissue prepared anhydrously in organic-solvents. J Ultra Mol Struct R 59(1):1–30. doi:10.1016/S0022-5320(77)80025-7

Legeros RZ (1974) Variations in crystalline components of human dental calculus. 1. Crystallographic and spectroscopic methods of analysis. J Dent Res 53(1):45–50

LeGeros RZ (1991) Calcium phosphates in oral biology and medicine, vol 15, Monographs in oral science. Karger, Basel

Legeros RZ, Pan CM, Suga S et al (1985) Crystallo-chemical properties of apatite in atremate brachiopod shells. Calcif Tiss Int 37(1):98–100. doi:10.1007/Bf02557687

Lehninger AL (1970) Mitochondria and calcium ion transport: fifth jubilee lecture. Biochem J 119(2):129–138

Leveque I, Cusack M, Davis SA et al (2004) Promotion of fluorapatite crystallization by soluble-matrix proteins from *Lingula anatina* shells. Angew Chem Int Edit 43(7):885–888. doi:10.1002/anie.200353115

Lie T, Selvig KA (1974) Calcification of oral bacteria – an ultrastructural study of 2 strains of *Bacterionema matruchotii*. Scand J Dent Res 82(1):8–18

Linton KM, Tapping CR, Adams DG et al (2013) A silicon cell cycle in a bacterial model of calcium phosphate mineralogenesis. Micron 44:419–432. doi:10.1016/j.micron.2012.09.008

Lorch IJ (1949a) Alkaline phosphatase and the mechanism of ossification. J Bone Joint Surg Br 31(1):94–99

Lorch IJ (1949b) The distribution of alkaline phosphatase in relation to calcification in *Scyliorhinus canicula* – development of the endoskeleton. Q J Microsc Sci 90(4):381–390

Lorch J (1949c) The distribution of alkaline phosphatase in the skull of the developing trout. Q J Microsc Sci 90(2):183–206

Lowenstam H (1972) Phosphatic hard tissues of marine invertebrates – their nature and mechanical function, and some fossil implications. Chem Geol 9(3):153–166. doi:10.1016/0009-2541(72)90053-8

Lowenstam HA (1981) Minerals formed by organisms. Science 211(4487):1126–1131. doi:10.1126/science.7008198

Lowenstam HA, Margulis L (1980) Evolutionary prerequisites for early phanerozoic calcareous skeletons. Biosystems 12(1–2):27–41, http://dx.doi.org/10.1016/0303-2647(80)90036-2

Lowenstam HA, Weiner S (1983) Mineralization by organisms and the evolution of Biomineralization. In: Biomineralization and biological metal accumulation. D. Reidel Publishing Co., Boston U.S.A., pp 191–203

Lowenstam HA, Weiner S (1989) On biomineralization. Oxford University Press, New York

Lynn WS, Brown RH (1963) Synthesis of polyphosphate by rat liver mitochondria. Biochem Biophys Res Co 11(5):367. doi:10.1016/0006-291x(63)90124-4

Mahamid J, Sharir A, Gur D et al (2011) Bone mineralization proceeds through intracellular calcium phosphate loaded vesicles: a cryo-electron microscopy study. J Struct Biol 174(3):527–535. doi:10.1016/j.jsb.2011.03.014

Margulis L (1981) Symbiosis in cell evolution: life and its environment on the early earth. Freeman, San Francisco

Martland M, Robison R (1924) The possible significance of hexosephosphoric esters in ossification. Part V. The enzyme in the early stages of bone development. Biochem J 18(6):1354–1357

McConnell D (1973) Apatite: its crystal chemistry, mineralogy, utilization, and geologic and biologic occurrences, vol 5, Applied mineralogy. Springer, New York

McCullough JF, van Wazer JR, Griffith EJ (1956) Structure and properties of the condensed phosphates. XI. Hydrolytic degradation of Graham's salt. J Am Chem Soc 78(18):4528–4533. doi:10.1021/ja01599a006

McGee-Russell SM (1955) A new reagent for the histochemical and chemical detection of calcium. Nature 175(4450):301–302

Meloan SN, Puchtler H (1985) Chemical mechanisms of staining methods: Von Kossa's technique: what von Kossa Really wrote and a modified reaction for selective demonstration of inorganic phosphates. J Histotechnol 8(1):11–13. doi:10.1179/his.1985.8.1.11

Millán JL (2006) Alkaline phosphatases: structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. Purinergic Signal 2(2):335–341. doi:10.1007/s11302-005-5435-6

Molnar Z (1959) Development of the parietal bone of young mice. 1. Crystals of bone mineral in frozen-dried preparations. J Ultra Mol Struct R 3(1):39–45. doi:10.1016/S0022-5320(59)80012-5

Moorer WR, Tencate JM, Buijs JF (1993) Calcification of a cariogenic *Streptococcus* and of *Corynebacterium* (*Bacterionema*) *matruchotii*. J Dent Res 72(6):1021–1026. doi:10.1177/00220345930720060501

Morse JW (1974) Dissolution kinetics of calcium-carbonate in sea-water. 3. New method for study of carbonate reaction-kinetics. Am J Sci 274(2):97–107
Mudd S, Yoshida A, Koike M (1958) Polyphosphate as accumulator of phosphorus and energy. J Bacteriol 75(2):224–235
Muhammed A (1959) The metabolism of phosphate polymers in micro-organisms. Oxford University, Oxford
Nathan Y, Bremner JM, Lowenthal RE et al (1993) Role of bacteria in phosphorite genesis. Geophys J Roy Astron Soc 11(2):69–76
Nieland ML, Von Brand T (1969) Electron microscopy of Cestode calcareous corpuscle formation. Exp Parasitol 24(2):279–289. doi:10.1016/0014-4894(69)90166-0
Nilsson JR, Coleman JR (1977) Calcium-rich, refractile granules in *Tetrahymena pyriformis* and their possible role in intracellular ion-regulation. J Cell Sci 24:311–325
O'Brien GW, Harris JR, Milnes AR et al (1981) Bacterial origin of east Australian continental-margin phosphorites. Nature 294(5840):442–444. doi:10.1038/294442a0
Omelon SJ, Grynpas MD (2008) Relationships between polyphosphate chemistry, biochemistry and apatite biomineralization. Chem Rev 108(11):4694–4715. doi:10.1021/cr0782527
Omelon S, Grynpas M (2011) Polyphosphates affect biological apatite nucleation. Cells Tissues Organs 194(2–4):171–175. doi:10.1159/000324845
Omelon S, Ariganello M, Bonucci E et al (2013) A review of phosphate mineral nucleation in biology and geobiology. Calcif Tiss Int 93(4):382–396. doi:10.1007/s00223-013-9784-9
Omelon S, Georgiou J, Variola F et al (2014) Colocation and role of polyphosphates and alkaline phosphatase in apatite biomineralization of elasmobranch tesserae. Acta Biomater 10(9): 3899–3910. doi:10.1016/j.actbio.2014.06.008
Omelon S, Georgiou J, Habraken WJEM (2016) A cautionary (spectral) tail: red-shifted fluorescence by DAPI–DAPI interactions. Biochem Soc Trans 44(1):46–49. doi:10.1042/bst20150231
Ooi SW, Smillie AC, Kardos TB et al (1981) Intracellular mineralization of *Bacterionema matruchotii*. Can J Microbiol 27(3):267–270
Osborn D, Hamilton TC (1977) Electron microbeam analysis of calcium distribution in Ciliated Protozoan, *Spirostomum ambiguum*. J Cell Physiol 91(3):409–416. doi:10.1002/jcp.1040910311
Pace NR (2006) Time for a change. Nature 441(7091):289–289. doi:10.1038/441289a
Padmavathi PB (1958) Histochemical evidence for the presence of phosphatase activity in spirostomum ambiguum. Naturwissenschaften 45(22):553–553. doi:10.1007/bf00632078
Pan CM, Watabe N (1988a) Shell growth of *Glottidia pyramidata* Stimpson (Brachiopoda, Inarticulata). J Exp Mar Biol Ecol 119(1):43–53. doi:10.1016/0022-0981(88)90151-7
Pan CM, Watabe N (1988b) Uptake and transport of shell minerals in *Glottidia pyramidata* Stimpson (Brachiopoda: Inarticulata). J Exp Mar Biol Ecol 118(3):257–268. doi:10.1016/0022-0981(88)90077-9
Pasteris JD, Yoder CH, Wopenka B (2014) Molecular water in nominally unhydrated carbonated hydroxylapatite: the key to a better understanding of bone mineral. Am Mineral 99(1):16–27. doi:10.2138/am.2014.4627
Pautard FGE (1958) Bone salts in unicellular organisms. Biochim Biophys Acta 28(3):514–520. doi:10.1016/0006-3002(58)90513-4
Pautard FGE (1959) Hydroxyapatite as a developmental feature of *Spirostomum ambiguum*. Biochim Biophys Acta 35(1):33–46. doi:10.1016/0006-3002(59)90332-4
Pautard FGE (1966) A biomolecular survey of calcification. In: Fleisch H, Blackwood HJJ, Owen M (eds) Calcified tissues 1965: proceedings of the third European symposium on calcified tissues held at Davos (Switzerland), April 11th–16th, 1965. Springer, Berlin, pp 108–122. doi:10.1007/978-3-642-85841-3_22
Pautard FGE (1970) Calcification in unicellular organisms. In: Schraer H (ed) Biological calcification: cellular and molecular aspects. Springer US, Boston, pp 105–201. doi:10.1007/978-1-4684-8485-4_4
Pautard FGE (1981) Calcium-phosphate microspheres in biology. Prog Cryst Growth Charact 4(1–2):89–98. doi:10.1016/0146-3535(81)90049-6

Poff AM, Pearce EIF, Larsen MJ et al (1997) Human supragingival in vivo calculus formation in relation to saturation of saliva with respect to calcium phosphates. Arch Oral Biol 42(2):93–99. doi:10.1016/S0003-9969(96)00120-3

Policard A (1929) La microincineration des cellules et des tissus. Protoplasma 7(1):464–481

Policard A (1942) Twenty years of microincineration. J Roy Microscop Soc 62(1–2):25–35. doi:10.1111/j.1365-2818.1942.tb00898.x

Posner AS (1969) Crystal chemistry of bone mineral. Physiol Rev 49(4):760–792

Quekett J (1849) On the intimate structure of bone, as composing the skeleton, in the four great classes of animals, viz., mammals, birds, reptiles, and fishes, with some remarks on the great value of the knowledge of such structure in determining the affinities of minute fragments of organic remains. Trans Microsc Soc Lond 2(1):46–58

Rao NN, Gomez-Garcia MR, Kornberg A (2009) Inorganic polyphosphate: essential for growth and survival. Annu Rev Biochem 78:605–647. doi:10.1146/annurev.biochem.77.083007.093039

Rey C, Combes C, Drouet C et al (2009) Bone mineral: update on chemical composition and structure. Osteoporos Int 20(6):1013–1021. doi:10.1007/s00198-009-0860-y

Robison R (1923) The possible significance of hexosephosphoric esters in ossification. Biochem J 17(2):286–293

Rosenberg H (1966) Isolation and identification of volutin granules from Tetrahymena. Exp Cell Res 41(2):397–410. doi:10.1016/S0014-4827(66)80147-7

Schewiakoff W (1894) Über die natur der sogenannten exkretkörner der Infusorien. Ziet Wiss Zool 57:32–56

Schulz HN, Schulz HD (2005) Large sulfur bacteria and the formation of phosphorite. Science 307(5708):416–418. doi:10.1126/science.1103096

Scott DB, Nylen MU, von Brand T et al (1962) The mineralogical composition of the calcareous corpuscles of *Taenia taeniaeformis*. Exp Parasitol 12(6):445–458, http://dx.doi.org/10.1016/0014-4894(62)90081-4

Shapiro IM, Greenspan JS (1969) Are mitochondria directly involved in biological mineralisation. Calc Tiss Res 3(1):100–102. doi:10.1007/Bf02058651

Simkiss K (1964) Phosphates as crystal poisons of calcification. Biol Rev 39(4):487–505. doi:10.1111/j.1469-185X.1964.tb01166.x

Simkiss K (1976) Intracellular and extracellular routes in biomineralization. In: Symposium of the society of experimental biology, pp 423–444

Simkiss K (1980) Detoxification, calcification and the intracellular storage of ions. The mechanisms of biomineralization in animals and plants. Tokai University Press, Tokyo, pp 13–18

Skinner HCW (1989) Low temperature carbonate phosphate materials or the carbonate—apatite problem: a review. In: Crick RE (ed) Origin, evolution, and modern aspects of biomineralization in plants and animals. Springer, New York, pp 251–264

Sundberg M, Friskopp J (1985) Crystallography of supragingival and subgingival human dental calculus. Scand J Dent Res 93(1):30–38

Takazoe I, Nakamura T (1965) The relation between metachromatic granules and intracellular calcification of *Bacterionema matruchotii*. Bull Tokyo Dent Coll 35:29–42

Termine JD, Peckausk R, Posner AS (1970) Calcium phosphate formation in-vitro. 2. Effects of environment on amorphous-crystalline transformation. Arch Biochem Biophys 140(2):318–325. doi:10.1016/0003-9861(70)90072-X

Thomas RS, Greenawalt JW (1968) Microincineration electron microscopy and electron diffraction of calcium phosphate-loaded mitochondria. J Cell Biol 39(1):55–76. doi:10.1083/jcb.39.1.55

Tyusenkov AS, Cherepashkin SE (2014) Scale inhibitor for boiler water systems. Russ J Appl Chem 87(9):1240–1245. doi:10.1134/S1070427214090080

Van Wazer JR (1958) Phosphorus and its compounds, vol 1. Interscience, New York

Van Wazer JR, Campanella DA (1950) Structure and properties of the condensed phosphates 4. Complex ion formation in polyphosphate solutions. J Am Chem Soc 72(2):655–663

Van Wazer JR, Griffith EJ, McCullough JF (1955) Structure and properties of the condensed phosphates. VII. Hydrolytic degradation of pyropolyphosphate and tripolyphosphate. J Am Chem Soc 77(2):287–291. doi:10.1021/ja01607a011

von Brand T, Mercado TI, Nylen MU et al (1960) Observations on function, composition, and structure of cestode calcareous corpuscles. Exp Parasitol 9(3):205–214, http://dx.doi.org/10.1016/0014-4894(60)90026-6

von Brand T, Scott DB, Nylen MU, Pugh MH (1965) Variations in mineralogical composition of Cestode calcareous corpuscles. Exp Parasitol 16(3):382–391. doi:10.1016/0014-4894(65)90061-5

von Brand T, Nylen MU, Martin GN, F.K. C (1967) Composition and crystallization patterns of calcareous corpuscles of Cestodes grown in different classes of hosts. J Parasitol 53(4):683–687. doi:10.2307/3276750

von Brand T, Nylen MU, Martin GN et al (1969) Cestode calcareous corpuscles: phosphate relationships, crystallization patterns, and variations in size and shape. Exp Parasitol 25:291–310

Watabe N (1989) Calcium phosphate structures in invertebrates and protozoans. In: Carter JG (ed) Skeletal biomineralization: patterns, processes and evolutionary trends. Springer, New York, pp 35–44. doi:10.1029/SC005p0035

Watabe N, Pan CM (1984) Phosphatic shell formation in atremate brachiopods. Am Zool 24(4):977–985

Watabe N, Wilbur KM (1976) The mechanisms of mineralization in the invertebrates and plants: symposium. In: Belle W (ed) Baruch Institute for Marine Biology and Coastal Research

Weinbach EC, von Brand T (1967) Formation isolation and composition of dense granules from mitochondria. Biochim Biophys Acta 148(1):256–266. doi:10.1016/0304-4165(67)90301-7

Weinbach EC, Vonbrand T (1965) Isolation and composition of dense granules from Ca++-loaded mitochondria. Biochem Biophys Res Commun 19(1):133–137. doi:10.1016/0006-291x(65)90131-2

Weiner S, Addadi L (2011) Crystallization pathways in biomineralization. Annu Rev Mater Res 41:21–40. doi:10.1146/annurev-matsci-062910-095803

Weiner S, Wagner HD (1998) The material bone: structure mechanical function relations. Annu Rev Mater Sci 28:271–298. doi:10.1146/annurev.matsci.28.1.271

Williams A, Mackay S, Cusack M (1992) Structure of the organo-phosphatic shell of the Brachiopod Discina. Philos T Roy Soc B 337(1279):83–104. doi:10.1098/rstb.1992.0086

Wuthier RE, Russell RGG, Bisaz S, Fleisch H (1972) Relationship between pyrophosphate, amorphous calcium phosphate and other factors in sequence of calcification in-vivo. Calc Tiss Res 10(3):198–206. doi:10.1007/Bf02012549

Zander HA, Hazen SP, Scott DB (1960) Mineralization of dental calculus. P Soc Exp Biol Med 103(2):257–260

Zhang F, Blasiak LC, Karolin JO et al (2015) Phosphorus sequestration in the form of polyphosphate by microbial symbionts in marine sponges. Proc Natl Acad Sci U S A 112(14):4381–4386. doi:10.1073/pnas.1423768112

Inorganic Polyphosphate Is an Essential Structural and Functional Component of the Mammalian Ion Channel TRPM8

13

Eleonora Zakharian

Abstract

Inorganic polyphosphate (polyP) is the largest inorganic macromolecule known in living organisms. The presence of polyP in the volcanic environment is indicative of its essential contribution to prebiotic evolution. On the other hand, the ubiquitous expression of polyP in higher eukaryotes suggests its important physiologic roles. Therefore, a question of whether polyP is a "molecular fossil" that can be viewed as a trace material from an evolutionary perspective, or, perhaps, polyP is implicated in the critical physiological processes, is yet to be determined. However, not only the ubiquitous presence of this polymer at all levels of the living hierarchy but also its expression pattern in the critical life organs like the brain, heart, liver, etc. indicates its indispensible physiological function. In this chapter, I review one of the most beautiful functions of polyP, its contribution to the formation of ion channels. This role of polyP is conditioned by its physicochemical nature. This to be said that polyP is a highly negatively charged polymorphic homopolymer, composed of tens or hundreds of phosphate residues linked by high-energy phosphoanhydride bonds. The multiple charges along the polyP's backbone create a high capacity for ion exchange with a preference for binding multivalent cations, attributed to their higher binding energies. For instance, Ca^{2+} and polyP thus make stable salt formation, which can subsequently be solubilized by another mysterious polymer, polyhydroxybutyrate (PHB). PHB is a homopolymer, comprised of ketone bodies β-hydroxybutyrate. Incorporation of the PHB-polyP-Ca^{2+} complex into the membrane creates an ideally Ca^{2+}-selective channel, where the cations move along the polymers, when driving force in the form of membrane potential or ion gradient is applied. The nuances of this polymeric channel formation and its association with ion channel proteins are the focus of this chapter.

E. Zakharian
Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine, 1 Illini Dr., Peoria, IL 61605, USA
e-mail: zakharel@uic.edu

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_13

13.1 Introduction

Inorganic polyphosphate (polyP) is the largest inorganic macromolecule known in living organisms. PolyP is a linear homopolymer consisting of orthophosphate residues linked by high-energy phosphoanhydride bonds (Fig. 13.1) (Kornberg 1995). In mammals the polymers of polyP vary greatly in their length in a tissue- or organ-specific manner (Gabel and Thomas 1971). The diversity in polyP amounts and lengths in various tissues suggests multiplicity of specific roles amended by this polyanion (Kumble and Kornberg 1995), although many of them yet remain elusive. The most anticipated polyP contribution would be accounted for its high-energy bonds and highly negative charge density carried by the polymer. Indeed, the molecular structure of polyP creates a high capacity for ion exchange with a preference for binding multivalent cations, attributed to their higher binding energies (Majling and Hanic 1980).

Polyphosphate is ubiquitous and implicated in diverse functions (Kumble and Kornberg 1995, 1996; Kornberg et al. 1999). In prokaryotes, polyP plays central roles in many general physiological processes, such as acting as a reservoir of energy and phosphate, as a chelator of metals, and as a buffer against alkali, in physiological adjustments to growth, as well as to stress response (Kornberg 1995). In higher eukaryotes, polyP contributes to the stimulation of mammalian target of rapamycin, involved in the proliferation of mammary cancer cells (Wang et al. 2003), and regulates mitochondrial function (Abramov et al. 2007). One of the critical roles of polyP that has recently emerged is its direct involvement in the maintenance of blood coagulation (Morrissey 2012). It is extraordinary that polyP is not only implicated in the coagulation cascade at various steps of coagulation factors activation but directly interacts with the fibrin clot and significantly thickens the fibrin mesh in a concentration-dependent manner (Smith and Morrissey 2008). However, many aspects of polyP function in mammalian organisms yet remain to be uncovered.

In biophysical perspective of polyP actions, one of the most attracting polyP roles is its participation in ion transport. In the association with a solvating amphiphilic polymer poly-(R)-3-hydroxybutyrate (PHB), polyP is capable of forming ion channels with a high selectivity for cations (Reusch et al. 1995). Channel-forming polyP-PHB-Ca^{2+} complexes have been found in bacterial and mitochondrial membranes (Das et al. 1997; Pavlov et al. 2005; Zakharian and Reusch 2007). Furthermore, polyP and PHB are associated with a variety of membrane proteins and are required for their normal function Zakharian and Reusch 2007; Negoda et al. 2007).

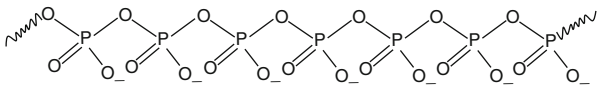

Fig. 13.1 Structure of inorganic polyphosphate (polyP). Phosphate residues with monovalent charge joined by flexible phosphoanhydride bonds

In this chapter I focus on the particular role of polyP in the formation of complexes with a specific class of proteins, called ion channels. I describe the formation of such a complex with the mammalian ion channel TRPM8.

13.2 PHB and PolyP Form Ion Channels

It has been well established that both PHB and polyP are capable of forming Ca^{2+} channels due to their intrinsic capacities for cation selection and transport (Reusch et al. 1995; Das et al. 1997; Seebach and Fritz 1999). This unique discovery on the formation of PHB/polyP ion channels was made by an outstanding physical chemist and microbiologist Dr. Rosetta N. Reusch. She determined that PHB/polyP complexes form cation-selective channel and also posttranslationally modify a number of bacterial ion channels and porins. How is this complex structured and how it can contribute to ion conduction? This question can be answered by looking into the molecular structure of both polymers. PHB is comprised of hydrophobic methyl groups alternating with hydrophilic ester oxygen groups (Fig. 13.2). In its molecular structure, PHB forms helices, where the ester oxygens of the polymer are constrained towards the inside of the helix, thus, creating a highly hydrophilic internal core, while its methyl groups extend outwards, covering the polymer with an entirely lipophilic surface (Fig. 13.3) (Seebach and Fritz 1999). This molecular structure of PHB makes it an excellent solvent for the polyP salts. In other words, PHB creates a shell wrapping around polyP. Remarkably, both polymers are held together by cations that bridge them along the core. The ester carbonyl oxygens of PHB and the phosphoryl oxygens of polyP create binding cavities with a ligand geometry particularly suitable for binding calcium ions (Fig. 13.3). This complex readily incorporates into the membrane, and application of voltage or ion gradient stimulates the ion flow along the track (Reusch and Sadoff 1988; Reusch 1989; Reusch et al. 1995; Seebach and Fritz 1999). Such complexes of PHB-polyP-Ca^{2+} channels have been found in bacterial and mitochondrial membranes (Reusch et al. 1995; Das et al. 1997; Pavlov et al. 2005), and their association with bacterial channels and porins is required for normal functioning of their hosts (Zakharian and Reusch 2007; Negoda et al. 2007, 2009).

In 2009, we discovered that the mammalian ion channel, from the transient receptor potential melastatin family, TRPM8 is modified at the posttranslational level with both the polymers, PHB and polyP (Zakharian et al. 2009). For the Ca^{2+}-permeable cation channel like TRPM8, this modification was indicative of a formation of the supramolecular complex, in which the protein, PHB, and polyP are

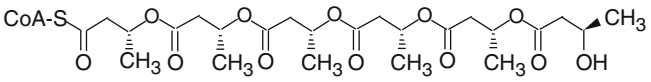

Fig. 13.2 Structure of poly-(R)-3-hydroxybutyrate (PHB) with the CoA-ester binding group and hydrophobic methyl groups alternating with hydrophilic ester carbonyl oxygens

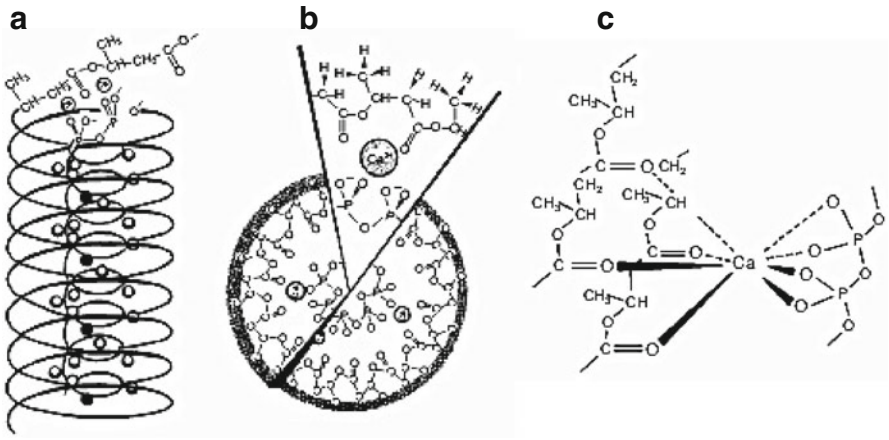

Fig. 13.3 PHB, polyP, and Ca^{2+} in the formation of ion channels. (**a**) PHB forms a helix with a lipophilic shell of methyl groups and polar lining of ester carbonyl oxygens surrounding a core helix of polyP with Ca^{2+} bridging the two polymers. (**b**) A *top* view at the channel structure. (**c**) Coordination of Ca^{2+} by polyP and PHB. Ca^{2+} forms ionic bonds with four phosphoryl oxygens of polyP and coordinate bonds with four ester carbonyl oxygens of PHB to form a neutral complex with irregular cubic geometry (Reproduced from Reusch 1989)

engaged in the functional integrity for ion transport. Previously, such complexes have been identified in bacteria. One of the exciting models within these protein/polyP/PHB complexes has been suggested for a potassium channel of *Streptomyces lividans* KcsA (Reusch 1999; Hegermann et al. 2008). Intriguingly, the association of polyP with KcsA was sown to contribute to the critical ion channel properties such as ion selectivity and gating mechanism (Zakharian and Reusch 2004; Negoda et al. 2007). These important propositions for the role of polyP complexed to a bacterial ion channel motivated us to investigate in details its designation with a similar mammalian complex on the example of TRPM8. This perspective was furthermore worthwhile of attention due to the significant physiological roles of the TRPM8 channel itself.

13.3 Physiological Roles of TRPM8

TRPM8 plays the role of a cold, menthol, and pain receptor in the peripheral nervous system McKemy et al. 2002; Peier et al. 2002). The cold perception mediated through TRPM8 was considered the primary function of this ion channel since 2002. Initially, however, the protein was found in the prostate epithelium (Tsavaler et al. 2001). Since then, although the role of TRPM8 as a cold receptor has been well established in the nervous system, the reasoning for the presence of a cold receptor in the prostate epithelium was not well justified. Recently, we pioneered in the field by identifying a novel role for TRPM8 as an ionotropic testosterone receptor (Asuthkar et al. 2015a, b). This fundamental discovery offered new directions in

studying the TRPM8 channel in the context of the androgen-dependent prostate tissues. Lately, we discovered that testosterone-induced TRPM8 activity presents a critical antitumor defense mechanism in prostate cancer (Asuthkar et al. 2015c). Furthermore, we found that prostate cancer cells eliminate their active Ca^{2+} uptake elements by avidly degrading the TRPM8 protein from the plasma membrane (Asuthkar et al. 2015c). Together these findings indicate that TRPM8 plays system-defined specific roles. In the context of the peripheral nervous system, TRPM8 is an important cold and pain receptor, while in the prostate epithelium the channel is a critical testosterone receptor that regulates Ca^{2+} homeostasis. Appreciating the physiological significance of TRPM8, the notion of its posttranslational modifications would not only benefit our understanding of the molecular mechanism for channel gating but also could present a valuable asset for possible therapeutic interventions.

13.4 Role of PolyP in TRPM8 Channel Function

Identifying the presence of polyP on the TRPM8 protein raised a question of whether the polyanion is implicated in the channel function. To evaluate polyP's role in TRPM8 function, we tested the channel activity upon polyP hydrolysis with exopolyphosphatase of *Saccharomyces cerevisiae* (scPPX1). Importantly, menthol-induced TRPM8 activity was significantly inhibited as during acute polyP removal, as well as in case of coexpressing scPPX1 with the channel (Zakharian et al. 2009). To further understand this inhibitory effect of polyP removal from TRPM8, we aimed its characterization at a single molecular level in planar lipid bilayers. The hydrolysis of polyP from TRPM8 resulted in an immense alteration in channel function (Figs. 13.4 and 13.5). However, most intriguingly was the fact that it affected both – the channel open probability and the conductance.

Within the first 5–7 min of scPPX1 treatment, a striking change was observed in the voltage dependence of TRPM8, where the voltage sensitivity was sharply converted from slight to strong (Fig. 13.4). Then, the open probability gradually decreased within ~30 min, taking in account time-dependent polyP hydrolysis by scPPX1, performed at room temperature. Simultaneously, within the same amount of time, the conductance decline was observed (Fig. 13.5). Interpreting these remarkable changes in TRPM8 channel behavior and properties suggested that polyP plays a critical role in the channel function. The effect of polyP on open probability and voltage dependence indicates that the polyanion is involved in the channel gating mechanism and its characteristic voltage sensitivity (Voets et al. 2007). The drop in conductance exerted by scPPX1 could be due to its blocking effect. ScPPX1 is an exopolyphosphatase, meaning that it removes orthophosphates from the terminal residue of the polymer, and as it continuously "chews up" polyP at its end, it could get to the proximity of the pore region and as a result occlude the ion flow. Together these findings indicated that polyP is an essential structural component of TRPM8 that plays a critical role in its function.

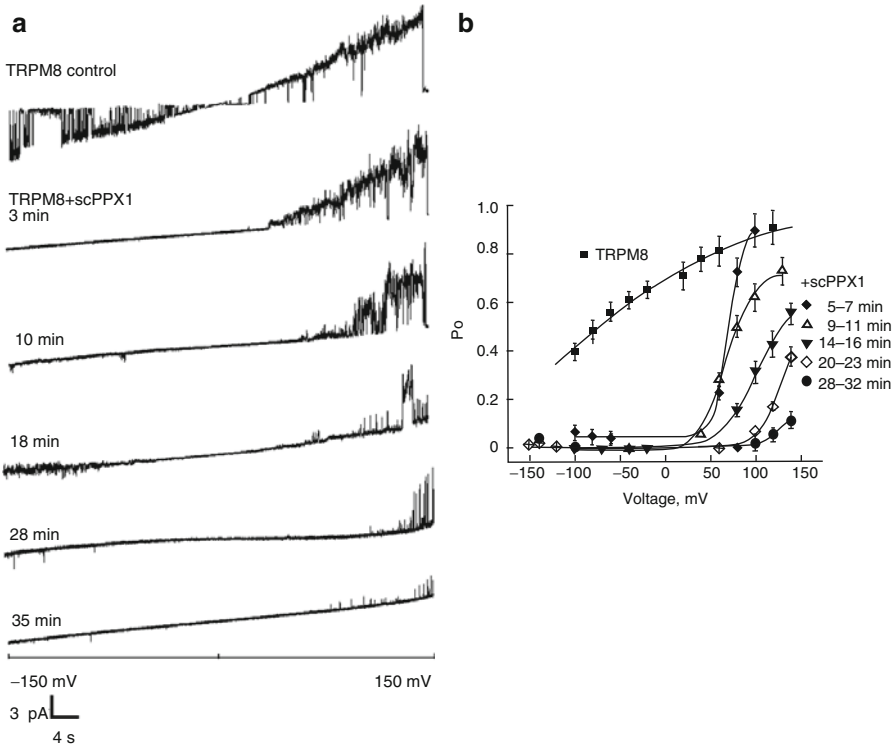

Fig. 13.4 PolyP is essential in voltage sensitivity of TRPM8. Voltage dependence of TRPM8 before and after the treatment with scPPX1: (**a**) Representative current traces recordings obtained at −150 + 150 mV voltage ramps before and after the treatment with polyphosphatase in a time course at the beginning of 3rd, 10th, 18th, 28th, and 33rd minutes. (**b**) The changes in open probability obtained at different voltages in gap-free recordings for TRPM8 alone (■) or after the treatment with scPPX1 for the following intervals of time: 5–7 min (♦), 9–11 min (Δ), 14–16 min (▼), 20–23 min (◊), and 28–32 min (●). Data were analyzed from overall of 16 experiments (The figure is reproduced from Zakharian et al. (2009), with the authorship rights preserved)

13.5 The Nature of PolyP Interaction with the TRPM8 Protein

The biochemical analysis of TRPM8 revealed that polyP associates with the protein by forming ionic bonds. This type of interaction became evident after evaluating TRPM8-polyP migration on the gel. We identified the presence of polyP only on TRPM8 tetramers detected in native PAGE and not SDS-PAGE. Unlike polyP, PHB was present on TRPM8 both under the native and denatured conditions, indicating that it bound to the protein covalently. Furthermore, the detailed mass spectrometric analysis identified various PHBylated peptides on the TRPM8 protein, supporting the covalent nature of this modification (Cao et al. 2013).

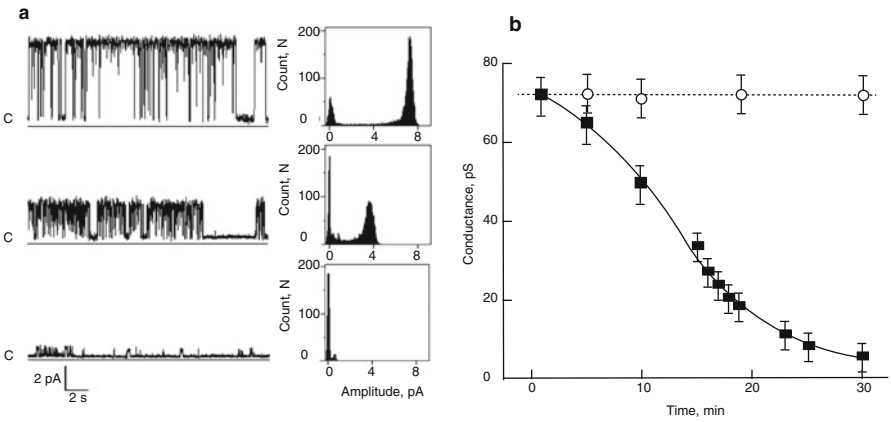

Fig. 13.5 Reduction of TRPM8 channel conductance by exopolyphosphatase scPPX1. (**a**) Representative single-channel current recordings of TRPM8 channels: upper traces – TRPM8 channel recordings before treatment with scPPX1; middle traces – TRPM8 channel recording 15 min later after the addition of scPPX1 (2 mg); lower traces – TRPM8 channel recordings after 30 min of addition of scPPX1. Clamping potential was +100 mV. (**b**) Symbols (■) ($n=5$) correspond to the mean conductance values of scPPX1 treated TRPM8 channels, where 2 mM of scPPX1 were added to the internal side of the channel; (○) ($n=8$) mean conductance of control, untreated channels (The figure is reproduced from Zakharian et al. (2009), with the authorship rights preserved)

Targeting the location of polyP interacting site, we identified that polyP is located on the internal side of the channel, according to the inhibitory effect exerted by scPPX1 that was effective only from the intracellular side. Although, the particular binding domain for polyP on TRPM8 has not been yet identified, it is conceivable that its interaction is mediated by PHB. Furthermore, considering that polyP contributes to the channel gating and voltage sensitivity and also the fact that scPPX1 is capable of blocking TRPM8 suggest that the complex may locate in the close proximity to the pore region.

13.6 Concluding Remarks

PolyP modifies the mammalian ion channel TRPM8 and plays an essential role in its channel function. Considering the tissue-specific roles of TRPM8 in the perception of the environment and regulation of the testosterone-dependent rapid signaling mechanism, the posttranslational modifications of the channel are, therefore, of great importance. Identified in our lab, two such modifications of the TRPM8 protein with PHB and polyP project their molecular integrity in forming functional ion channel complex and may have further use in the purpose of therapeutic intervention.

References

Abramov AY, Fraley C, Diao CT et al (2007) Targeted polyphosphatase expression alters mitochondrial metabolism and inhibits calcium-dependent cell death. Proc Natl Acad Sci U S A 104:18091–18096. doi:10.1073/pnas.0708959104

Asuthkar S, Elustondo PA, Demirkhanyan L et al (2015a) The TRPM8 protein is a testosterone receptor: I. Biochemical evidence for direct TRPM8-testosterone interactions. J Biol Chem 290:2659–2669

Asuthkar S, Demirkhanyan L, Sun X et al (2015b) The TRPM8 protein is a testosterone receptor: II. Functional evidence for an ionotropic effect of testosterone on TRPM8. J Biol Chem 290: 2670–2688

Asuthkar S, Velpula KK, Elustondo P et al (2015c) TRPM8 channel as a novel molecular target in androgen-regulated prostate cancer cells. Oncotarget

Cao C, Yudin Y, Bikard Y et al (2013) Polyester modification of the mammalian TRPM8 channel protein: implications for structure and function. Cell Rep 4:302–315. doi:10.1016/j.celrep.2013.06.022

Das S, Lengweiler UD, Seebach D et al (1997) Proof for a nonproteinaceous calcium-selective channel in Escherichia coli by total synthesis from (R)-3-hydroxybutanoic acid and inorganic polyphosphate. Proc Natl Acad Sci U S A 94:9075–9079

Gabel NW, Thomas V (1971) Evidence for the occurrence and distribution of inorganic polyphosphates in vertebrate tissues. J Neurochem 18:1229–1242

Hegermann J, Lunsdorf H, Overbeck J et al (2008) Polyphosphate at the *Streptomyces lividans* cytoplasmic membrane is enhanced in the presence of the potassium channel KcsA. J Microsc 229:174–182. doi:10.1111/j.1365-2818.2007.01863.x

Kornberg A (1995) Inorganic polyphosphate: toward making a forgotten polymer unforgettable. J Bacteriol 177:491–496

Kornberg A, Rao NN, Ault-Riche D (1999) Inorganic polyphosphate: a molecule of many functions. Ann Rev Biochem 68:89–125. doi:10.1146/annurev.biochem.68.1.89

Kumble KD, Kornberg A (1996) Endopolyphosphatases for long chain inorganic polyphosphate in yeast and mammals. J Biol Chem 271:27146–27151

Kumble KD, Kornberg A (1995) Inorganic polyphosphate in mammalian cells and tissues. J Biol Chem 270:5818–5822

Majling J, Hanic F (1980) Phase chemistry of condensed phosphates. Top Phosphorus Chem 10:341–502

McKemy DD, Neuhausser WM, Julius D (2002) Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416:52–58

Morrissey JH (2002) Polyphosphate: a link between platelets, coagulation and inflammation. Int J Hematol 95:346–352. doi:10.1007/s12185-012-1054-5

Morrissey JH (2012) Polyphosphate: a link between platelets, coagulation and inflammation. Int J Hematol 95, 346–352

Negoda A, Xian M, Reusch RN (2007) Insight into the selectivity and gating functions of Streptomyces lividans KcsA. Proc Natl Acad Sci U S A 104:4342–4346. doi:10.1073/pnas.0700495104

Negoda A, Negoda E, Xian M et al (2009) Role of polyphosphate in regulation of the *Streptomyces lividans* KcsA channel. Biochim Biophys Acta 1788:608–614. doi:10.1016/j.bbamem.2008.12.017

Pavlov E, Zakharian E, Bladen C et al (2005) A large, voltage-dependent channel, isolated from mitochondria by water-free chloroform extraction. Biophys J 88:2614–2625. doi:10.1529/biophysj.104.057281

Peier AM, Moqrich A, Hergarden AC et al (2002) A TRP channel that senses cold stimuli and menthol. Cell 108:705–715

Reusch RN (1989) Poly-beta-hydroxybutyrate/calcium polyphosphate complexes in eukaryotic membranes. Proc Soc Exp Biol Med 191:377–381

Reusch RN (1999) *Streptomyces lividans* potassium channel contains poly-(R)-3-hydroxybutyrate and inorganic polyphosphate. Biochemistry 38:15666–15672

Reusch RN, Sadoff HL (1988) Putative structure and functions of a poly-beta-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc Natl Acad Sci U S A 85:4176–4180

Reusch RN, Huang R, Bramble LL (1995) Poly-3-hydroxybutyrate/polyphosphate complexes form voltage-activated Ca^{2+} channels in the plasma membranes of *Escherichia coli*. Biophys J 69:754–766. doi:10.1016/S0006-3495(95)79958-1

Seebach D, Fritz MG (1999) Detection, synthesis, structure, and function of oligo(3-hydroxyalkanoates): contributions by synthetic organic chemists. Int J Biol Macromol 25:217–236

Smith SA, Morrissey JH (2008) Polyphosphate enhances fibrin clot structure. Blood 112:2810–2816. doi:10.1182/blood-2008-03-145755

Tsavaler L, Shapero MH, Morkowski S et al (2001) Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. Cancer Res 61:3760–3769

Voets T, Owsianik G, Janssens A et al (2007) TRPM8 voltage sensor mutants reveal a mechanism for integrating thermal and chemical stimuli. Nat Chem Biol 3:174–182. doi:10.1038/nchembio862

Wang L, Fraley CD, Faridi J et al (2003) Inorganic polyphosphate stimulates mammalian TOR, a kinase involved in the proliferation of mammary cancer cells. Proc Natl Acad Sci U S A 100:11249–11254. doi:10.1073/pnas.1534805100

Zakharian E, Reusch RN (2004) Functional evidence for a supramolecular structure for the *Streptomyces lividans* potassium channel KcsA. Biochem Biophys Res Commun 322:1059–1065. doi:10.1016/j.bbrc.2004.08.027

Zakharian E, Reusch RN (2007) *Haemophilus influenzae* outer membrane protein P5 is associated with inorganic polyphosphate and polyhydroxybutyrate. Biophys J 92:588–593. doi:10.1529/biophysj.106.095273

Zakharian E, Thyagarajan B, French RJ et al (2009) Inorganic polyphosphate modulates TRPM8 channels. PLoS One 4:e5404. doi:10.1371/journal.pone.0005404

Inorganic Polyphosphate in Tissue Engineering

14

Rahul Gawri*, Jean-Philippe St-Pierre*, Robert Pilliar, Marc Grynpas, and Rita A. Kandel

Abstract

Inorganic polyphosphates (polyP) are biopolymers of orthophosphates linked by phosphoanhydride bonds ubiquitously found in eukaryotic cells. In mammalian cells and tissues, they have been implicated in the regulation of a number of biological processes. Owing to their ease of processing into materials and broad range of biological effects, these inorganic polymers have been investigated for an increasing number of biomedical applications. This chapter reviews the use of inorganic polyphosphate in tissue engineering and regenerative medicine (TERM) applications. It's application in the fabrication of scaffold materials with interesting properties, mainly for bone repair but also to support tissue for-

* Both authors contributed equally to writing this chapter

R. Gawri
Institute of Biomaterial and Biomedical Engineering, University of Toronto, Toronto, ON, Canada

J.-P. St-Pierre
Lunenfeld Tannenbaum Research Institute, University of Toronto, Toronto, ON, Canada

R. Pilliar
Faculty of Dentistry, University of Toronto, Toronto, ON, Canada

M. Grynpas
Institute of Biomaterial and Biomedical Engineering, University of Toronto, Toronto, ON, Canada

Lunenfeld Tannenbaum Research Institute, University of Toronto, Toronto, ON, Canada

Department of Pathology and Laboratory Medicine and Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada

R.A. Kandel (✉)
Department of Pathology and Laboratory Medicine and Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada
e-mail: rkandel@mtsinai.on.ca

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9_14

mation in other systems will be discussed. Applications in drug delivery will also be covered. Finally, recent efforts to harness the bioactivity of polyP toward improved tissue repair and regeneration will be mentioned along with emerging applications that hold tremendous promise. As our understanding of the varied roles of polyP in tissue homeostasis continues to evolve, we are likely to see an increase in the use of this ubiquitous biomolecule for TERM applications.

14.1 Introduction

Inorganic polyphosphates (polyP) are biomolecules composed of orthophosphate units linked by energy-rich phosphoanhydride bonds to form polymeric chains. These phosphate polymers can aptly be called "biopolymers" as they exhibit varied biological roles and modulate numerous biological processes in archaea, bacteria, and eukaryotes, the three members of the phylogenetic tree of life.

PolyP in nature are formed both abiotically and biotically. Abiotic forms of polyP are generated by heating orthophosphate at high temperatures under anhydrous conditions. Biotic forms of polyP are generated by enzymatic processes that include polyphosphate kinases 1 and 2 (PPK1 and PPK2) in prokaryotes and homologues. The enzyme(s) synthesizing polyP in mammalian cells largely remain to be identified. PolyP in vertebrate cells have a bimodal chain length distribution, with short- or long-chain pools (Griffin et al. 1965; Pisoni and Lindley 1992; Lorenz et al. 1997). Interestingly, these chain length profiles appear to be cell type specific and a function of cellular state. This was highlighted by Lorenz et al. (1997) who reported that inducing apoptosis in human promyelocytic leukemia cells (HL60) causes the loss of the pool of long-chain polyP (Lorenz et al. 1997). The report also demonstrates age-dependent changes in the content of long-chain polyP in rat brain and liver. Others have reported that polyP chain length dictates biological functions. This is exemplified by a study that shows that phosphate polymers with chains of at least 500 orthophosphate units are optimal for the activation of the contact pathway, while chains of approximately 100 orthophosphate units are optimal for the activation of factor V in blood (Smith et al. 2010). Further, studies have shown that polyP exhibit cell and tissue type-specific subcellular distributions. They are particularly concentrated in the nuclei and plasma membrane of rat liver cells (Griffin et al. 1965; Kumble and Kornberg 1995) but are found in the mitochondria (Kumble and Kornberg 1995; Lynn and Brown 1963; Pavlov et al. 2010) and lysosomes (Pisoni and Lindley 1992) in other cell types. Of interest, they are also present in the extracellular matrix of hypertrophic growth plate cartilage (Omelon et al. 2009) and articular cartilage (St- Pierre 2011). Taken together, these findings suggest an important level of complexity to the biological effects of polyP, an otherwise structurally simple molecule. Given their diverse roles in cell function, polyP have been used extensively for tissue repair.

This chapter discusses the use of polyP in tissue engineering and regenerative medicine (TERM). First, we will describe the development of bioactive glass/ceramic-based scaffolds with interesting properties based on the use of polyP as a

building block. Next, we will describe the approaches used to date to harness the tissue-specific bioactivities of polyP to stimulate tissue repair/regeneration or to modulate biological processes. As our understanding of the breadth of biological functions carried out by polyP is expanding, this chapter ends by highlighting a few particularly novel-promising uses for this inorganic biopolymer in tissue repair. Owing to the key roles played by polyP in biological processes, emerging efforts to exploit this chemically simple and easily processed, but biologically complex, molecule for TERM applications are poised to provide substantial benefits for patients.

14.2 Bioactive Glasses and Bioactive Glass-Ceramics

Bioactive glasses and glass-ceramics are advantageous as biomaterials because they can interact with the host tissue and be replaced by tissue over time at a rate matched with the degradation of the material (Baino et al. 2015). Various types of bioactive glasses and glass-ceramics have been described as scaffold material for use in filling bone (or other tissues) defects, as well as for a range of other tissue engineering applications. Hench and Wilson (1984) classified bioactive ceramics into four categories: (1) dense hydroxyapatite (HA) ceramics, (2) bioactive glasses, (3) bioactive glass-ceramics, and (4) bioactive composites (Hench and Wilson 1984).

14.2.1 Polyphosphate-Containing Bioactive Glasses for Regenerative Medicine Applications

Since the formulation of the first bioactive glass, Bioglass 45S5®, for use in bone void filling applications in war veterans by Larry Hench in 1969, the field of bioactive glass has expanded rapidly and now includes silicate-, borate-, and phosphate-based bioactive glasses Hench & Jones (2015). Phosphate-based bioactive glasses are considered a distinct category of materials despite the fact that some silica-based bioglasses, such as Bioglass 45S5®, contain low levels of phosphate (6%). Bioglasses containing phosphate release oligo-elements during solubilization/breakdown that are responsible for crystal nucleation and apatite layer formation on the surface of the materials, an effect that is considered the main factor responsible for the bioactivity of silicate-based bioglasses (Delahaye et al. 1998). Compared to silicate bioglasses, phosphate bioglasses are characterized by a decreased chemical durability due to differences in the tetrahedron structure of silicon and phosphorus. Condensed phosphate molecules come in three forms: (i) chains which are called polyphosphates, (ii) rings which are called metaphosphates, and (iii) branched or cross-linked molecules which are called ultraphosphates (Griffith 1995). There are various phosphate glass structures, such as ultraphosphate glasses (compositions in the range $(M_{2/\nu}O)x(P_2O_5)_{1-x}$, where $0 < x < 0.5$ and ν is the valence of the metal cation and with the atomic ratio of oxygen to phosphorus, i.e., $3.0 \leq O/P \leq 2.5$); these ultraphosphates are highly soluble and unstable in aqueous solutions and therefore will not be considered further in this chapter (Griffith 1995; Abou Neel et al. 2009). Metaphosphate glasses (compositions in the range $(M_{2/\nu}O)_{0.5}(P_2O_5)_{0.5}$

and with the atomic ratio of oxygen to phosphorus, O/P = 3.0) form a ring structure that is hydrolytically degraded slowly and will also not be discussed further (Griffith 1995; Abou Neel et al. 2009). PolyP (including pyrophosphate, compositions in the range $(M_{2/\nu}O)x(P_2O_5)_{1-x}$, where $x > 0.5$ and with the atomic ratio of oxygen to phosphorus, i.e., O/P >3) (Abou Neel et al. 2009) in the form of polyP glasses have been used in a number of different tissue engineering applications. These include bone repair (Lakhkar et al. 2015; Al Qaysi et al. 2015; Zheng et al. 2015; Abou Neel and Knowles 2008), skeletal muscle regeneration (Shah et al. 2005, 2014), and to facilitate neurite outgrowth in peripheral nerve injury (Kim et al. 2015; Bitar et al. 2004). The use of bioactive glasses for tissue engineering functions is limited by its relatively slow rate of degradation (silicate and borate glasses) and its brittleness (phosphate glasses). A number of different materials were developed including tricalcium phosphate (TCP), hydroxyapatite, and calcium polyP-based ceramics to overcome these limitations.

14.3 Calcium Polyphosphate Bioceramic Substrate for Tissue Engineering Applications

Glasses can be converted to ceramics by a number of fabrication processes, including reheating cooled glasses. Glass-ceramics have the advantage of retaining some of the properties of its glass precursors, depending on the fabrication process. Porous calcium polyphosphate (CPP) is one such ceramic that is generated from calcium monobasic monohydrate phosphate via several steps. It is calcined to form CPP which is then melted at high temperature. The amorphous glass frit is ground and then sieved to obtain particles of desired sizes and converted to CPP ceramic scaffolds by sintering at high temperatures. One of the advantages of CPP is the ability to tune its mechanical and degradation properties via doping with different ions, such as Sr^{2+} (Xie et al. 2012) and Gd^{3+} (Wang et al. 2015). These materials can be made by gravity sintering or additive manufacturing, such as 3D printing, to facilitate formation of porous forms with controlled shapes that can be personalized to the individual and the defect being repaired (Shanjani et al. 2013).

14.3.1 CPP in Bone Tissue Engineering

CPP was first developed as a bone substitute to address some of the shortcomings of traditional materials in use for bone healing such as hydroxyapatite (HA), tricalcium phosphate (TCP), and bioactive glasses. HA degrades relatively slowly, whereas the degradation rate of TCP is often overly rapid and less controllable (Wang et al. 2016a). CPP is an excellent bone substitute as it can be made with mechanical properties similar to cancellous bone, controlled biodegradability, and also shows good integration to the host bone when implanted in vivo (Grynpas et al. 2002, Pilliar et al. 2013, Hu et al. 2014). While CPP like all ceramics has limited ductility and exhibits brittle fracture behavior, methods are being developed to overcome these

characteristics. CPP has been used in different forms, such as sintered porous blocks (Pilliar et al. 2001, 2013, Hu et al. 2014), particulates (Pilliar et al. 2016) or nanoparticles (Chen et al. 2015). This flexibility in fabrication has made CPP a good biomaterial for use in tissue engineering. By far, the most common use of CPP has been in the field of bone repair. The literature on CPP in this field is extensive, and due to space limitations we can only highlight a few of the relevant studies. We wish to apologize to those authors whose papers have not been cited in this chapter.

Nelson et al. (1993) carried out one of the first investigations on CPP, in which they explored its ability to repair canine mandibular alveolar defects. The bone defect was either filled with CPP or autogenous bone. Evaluation at 4 months revealed increased bone and greater rates of union in the CPP group than in the bone graft control (Nelson et al. 1993). Our group has evaluated the properties of CPP porous implants extensively both in vitro and in vivo. In vitro studies have shown that CPP degrades in solutions that are either physiological (pH 7) or similar to that found in resorption pits produced by osteoclasts (pH 4), likely due to polyP hydrolysis (Pilliar et al. 2001). Studies in which porous CPP rods were placed in rabbit femoral defect sites showed that degradation was faster during the initial weeks postimplantation. Interestingly the rate of degradation was inversely proportional to the particle size used to form the CPP. It was also shown that the rate of degradation was one order magnitude higher in vivo than in vitro, probably due to the contribution of cell resorption. The 4 mm diameter rods were completely infiltrated by the bone within 6 weeks suggesting that the CPP particles forming the porous structure were osteoconductive (Grynpas et al. 2002). CPP porous implants made by additive manufacturing followed by sintering resulted in stronger porous implants and also allowed good bone ingrowth (Shanjani et al. 2013, Hu et al. 2014). Several modifications have been made to CPP to alter its biodegradability and compressive properties. One such commonly used technique is ion doping of CPP substrates. Ion doping introduces crystal defects and alters the degradation rate of CPP. Song et al. (2009) showed that doping with different ions affected degradation rate in the following order, $Mg^{2+} > Na^{+} > K^{+} > Zn^{2+} > Sr^{2+}$, and the authors suggested that when the doping element had a much smaller ion radius and equal valence compared to Ca^{2+}, it could significantly accelerate CPP degradation and mineralization. Conversely, the doping element with similar ion radius and different valence compared to Ca^{2+} could decrease degradation and mineralization rate (Song et al. 2009). Doping can also have other benefits. Studies have shown that Sr^{2+}-CPP accelerates bone healing in part by stimulating secretion of vascular endothelial growth factor (VEGF) and fibroblast growth factor beta (bFGF) by osteoblasts (Gu et al. 2013). It can also enhance macrophage proliferation and inhibit osteoclast function (Gu et al. 2012). Various other groups have made further modifications to K^{+}/Sr^{2+}-CPP, such as immobilizing silk fibroin on these doped materials, which resulted in further enhanced angiogenic and osteogenic properties (Wang et al. 2016a). Altering CPP in other ways can also influence the effect of CPP on bone healing. A recent study by Chen et al. (2016) evaluated the effect of CPP material with porosity gradients and reported that the presence of this feature elicited significant increases in mineralization and alkaline phosphatase expression by MC3T3-E1 cells compared to

isotropic CPP porous implants (Chen et al. 2016). The mechanism underlying this response is unknown. Other groups have developed antibiotic conjugated CPP, and Song et al. (2011), for example, showed that Sr^{2+}-doped CPP porous implants not only released the antibiotic but also could have additional effects as they observed that it also inhibited osteoclastogenesis by RAW264.7 cell line (Song et al. 2011). These varied responses emphasize the potential powerful effects polyP scaffolds may have once the optimized forms are identified.

CPP produced as particulate has been used to repair critical-sized bone defects in a rabbit model. CPP particulate facilitated defect bridging in a manner similar to commercially available TCP particulates. Interestingly defects filled with slow-degrading CPP (SD-CPPp) particulates contained more bone tissue than the defects filled with fast-degrading CPP particulates (FD-CPPp) (Fig. 14.1) (Pilliar et al. 2016). This suggests that controlling the rate of CPP degradation can help modulate bone ingrowth and/or formation.

CPP can also be used to in combination with other biomaterials to enhance their properties. For example, Zhou et al. (2012) used CPP in fiber form and mixed it with calcium phosphate cement with or without the micromorselized bone to create a bone void filler composite. The presence of CPP increased the mechanical strength and porosity of the composite. When implanted into rabbit bone defects, this

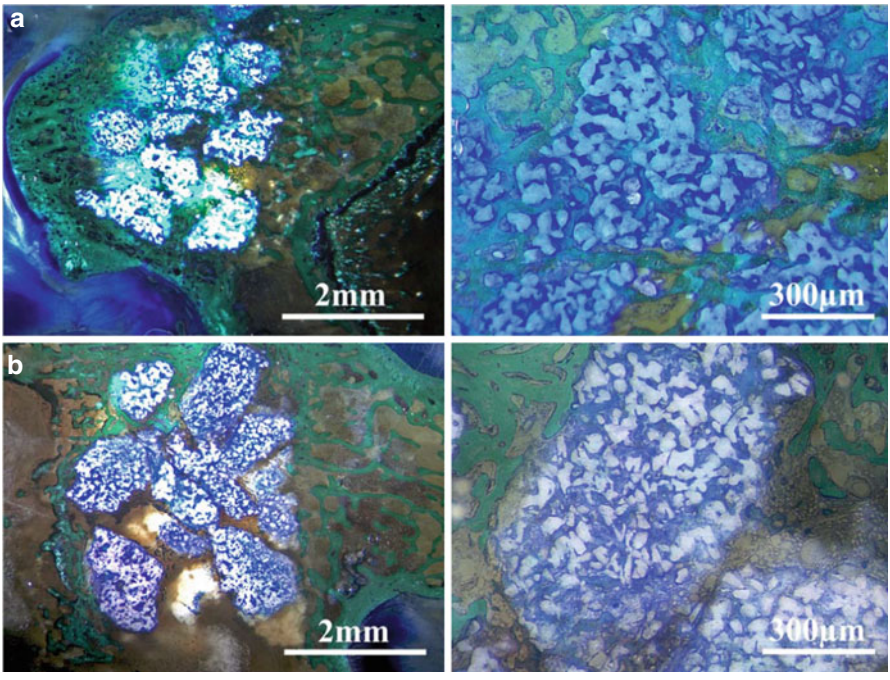

Fig. 14.1 Histology of 4-week implants: (**a**) SD-CPPp, (**b**) FD-CPPp – lower magnification on the left and higher magnification photomicrographs on the right; green-stained region = bone, blue-stained = nonmineralized tissue (Figure reproduced with permission Pilliar et al. (2016))

composite improved bone ingrowth compared to bone cement without CPP fibers (Zhou et al. 2012). These studies highlight the need to optimize polyP containing materials for bone tissue engineering applications.

14.3.2 CPP as Substrates for Interface Tissue Engineering

CPP has also been used to support the formation of tissues and as a bone substitute to help recreate the soft tissue-bone interface critical for restoring normal architecture and thus functionality for certain types of musculoskeletal tissues. This is a unique use of this porous implant.

14.3.2.1 Engineering Biphasic Osteochondral-Like Implants

Osteoarthritis is a disease of joints characterized by progressive degeneration of articular cartilage and changes in the underlying subchondral bone, causing pain and decreased range of motion in affected individuals. The etiology of this disease has not been fully delineated but can arise as a result of a sports injury that can start as focal cartilage erosion. There are no drugs currently available that can prevent disease progression, so there has been substantial interest in developing novel ways to regenerate or replace the damaged cartilage and underlying bone. One way to do this would be to create biphasic constructs to resurface the joint, which would have the added benefit of allowing for implant fixation through bone ingrowth within the porous structure of the bone substitute. CPP has been used as a bone substitute to create such "osteochondral-like" constructs. Chondrocytes isolated from the cartilage or derived from mesenchymal stromal cells (MSCs) are placed on the intended articulating surface of the CPP and grown in vitro in chondrogenic conditions to form a cartilage layer on and integrated with the porous CPP material to recreate the osteochondral organization. CPP has been shown to support the maintenance of the chondrocyte phenotype and formation of a cartilage-like tissue (Lee et al. 2015; Waldman et al. 2002). When biphasic constructs were implanted into focal osteochondral defects in sheep knees, they integrated well with the adjacent native cartilage and had comparable proteoglycan content to the native cartilage. The bone grew into the pores of the CPP and in some areas merged with the overlying cartilage-like layer (Fig. 14.2) (Kandel et al. 2006; Pilliar et al. 2007). Further, a zone of the calcified cartilage can be formed in vitro at the interface between the cartilage layer and CPP either with the use of chondrocytes enriched in the deep zone subpopulation or MSCs and selected culture conditions. However, this required the application of a sol-gel apatite "barrier" coating on CPP to decrease the release of polyP, which inhibits calcification at high concentrations (St-Pierre et al. 2010, 2012; Lee et al. 2015).

These biphasic "osteochondral-like" constructs have an additional use as they can be used as a model to test bioactive substances and supplements in vitro. For example, when these constructs were grown in culture in the presence of platelet-rich plasma (PRP), cartilage growth was enhanced and the tissues had better mechanical properties (Petrera et al. 2013). The use of these constructs to screen the

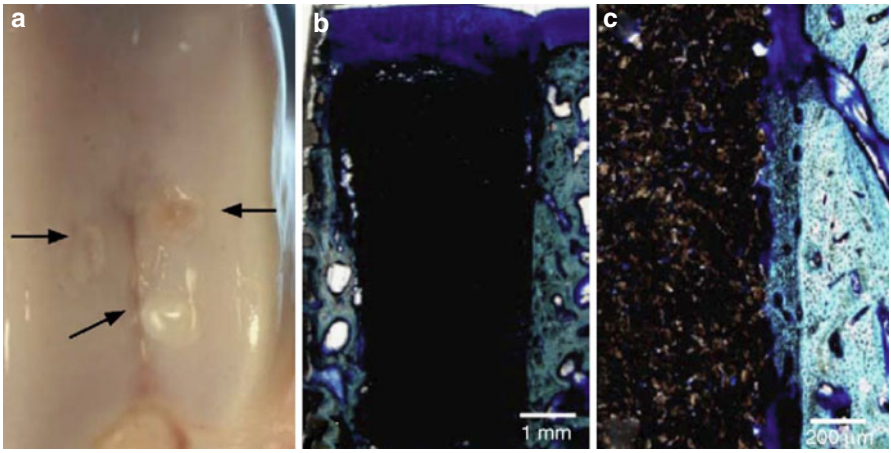

Fig. 14.2 (**a**) Gross appearance of biphasic implants at 9 months (→). (**b**) Histological appearance of a biphasic implant at 9 months. Hyaline cartilage can be seen overlying the CPP with fusion to the adjacent native cartilage. (**c**) At higher magnification, the bone can be seen abutting and growing into the CPP (toluidine blue stain) (Figure reproduced with permission from Kandel et al. (2006))

effect of bioactive agents may be a way to improve tissue-engineered cartilage prior to in vivo studies.

14.3.2.2 Engineering Intervertebral Disc Replacements Using CPP

The spine is composed of vertebral bodies with intercalating fibrocartilaginous structures known as intervertebral discs (IVDs). The IVD is composed of a nucleus pulposus (NP), which is surrounded by a multilamellated angle-ply annulus fibrosus (AF) tissue. These are integrated to the bone of the vertebral bodies in part through cartilage endplates. Degenerative disc disease (DDD) is associated with the development of neck or back pain and is ranked as the second most common cause of chronic non-life-threatening debilitating illnesses (after arthritis) (Cassidy et al. 1998). The degeneration can develop in the NP, AF, or cartilage endplate. As there is currently no optimal treatment for DDD, the development of strategies to replace parts or the entire IVD is an area of active investigation. CPP can be used as a bone substitute to replace the altered bone and as well to support IVD tissue formation. Séguin et al. (2004) placed isolated bovine NP cells on the porous CPP and the cells formed nucleus pulposus tissue on the top surface integrated to the CPP in a similar fashion to articular cartilage constructs. By 6 weeks, the bioengineered tissues showed extracellular matrix accumulation approximating native tissue levels of proteoglycan, the major macromolecule of this tissue. Tissue mechanical properties also improved over time (Seguin et al. 2004). Hamilton et al. (2006) used this approach to generate a triphasic NP-cartilage endplate-CPP bone substitute construct to mimic the native IVD architecture.

14.3.3 Engineering Dental Tissues Using CPP

There have been several studies examining tissue engineering of oral or dental tissues with CPP.

EI Sayegh et al. (2002) showed that the crystallinity and degradation rate of CPP did not substantially affect the interactions of human gingival fibroblasts with CPP materials but that compared with titanium alloy substrates, cell spreading and attachment were inhibited (EI Sayegh et al. 2002). Wang et al. (2006) seeded human dental pulp cells on CPP and demonstrated that they grew well within the 3D structure of the scaffolds. No significant difference was observed in cell viability compared to cells grown on HA and TCP scaffolds. These studies suggest that CPP has promise as a biomaterial for biological regenerative endodontic therapy (Wang et al. 2006).

14.3.4 Promoting Angiogenesis Using CPP

Angiogenesis is essential for the success of many tissue engineering applications. Identifying ways to promote this process is an area of intense investigation. In a study by Chen et al. (2008a, b), human umbilical vein endothelial cells (HUVECs) were grown on CPP or Sr^{2+}-CPP discs. The cells attached and spread better on Sr^{2+}-CPP than CPP (Chen et al. 2008b). Another study by Chen et al. (2008) using human umbilical vein endothelial cells grown on Sr^{2+}-CPP discs showed that these cells could form tubelike structures indicative of capillaries (Chen et al. 2008a). Others have shown that culture on Sr^{2+}-CPP discs stimulates cells to secrete angiogenic factors that could help drive neovascularization (Liu et al. 2011; Gu et al. 2013; Kang et al. 2015). Fu et al. (2015) found that Sr^{2+}-CPP discs supported tube formation by cocultures of peripheral blood-derived mesenchymal stem cells and endothelial progenitor cells suggesting a way to improve the vascularization of bone void fillers (Fu et al. 2015).

14.4 Drug Delivery Using CPP

CPP has also been used successfully as a drug delivery vehicle for immobilized therapeutic agents, which are released as the scaffold degrades. This functionality may be important to tissue engineering either as a way to prevent infection of implanted tissues or to treat infected fields into which the scaffold is placed. Schofield et al. (2006) studied the release of incorporated cefuroxime in gelled or ungelled CPP matrices. Gelled matrices showed slow release of the antibiotic over a period of 4 days at therapeutically relevant doses compared to ungelled matrices, which exhibited a burst release (Schofield et al. 2006). Several modifications to the gelled matrix have been proposed, such as adding a compaction step during the gelling protocol, which allowed for further fine tuning of the drug delivery profile by CPP matrices (Petrone et al. 2008). In a recent study, Ren et al. (2014) showed that

erythromycin doped Sr^{2+}-CPP placed in a mouse pouch infection model was bactericidal and was able to contain a low-grade *Staphylococcus aureus* infection (Ren et al. 2014). The biofunctionalization of CPP has far-reaching applications in augmenting the tissue response to the material through the supply of bioactive molecules such as growth factors. This strategy holds tremendous promise toward guiding repair across critical bone defects.

14.5 Harnessing the Bioactivity of Inorganic Polyphosphates for Tissue Engineering Applications

As described in other chapters in this book, polyP has a remarkable breadth of biological functions within the body, through complex and tightly regulated processes. These bioactivities can be exploited for improved tissue regeneration or to manage homeostasis and address pathologies. Sections 14.5 and 14.6 will address interesting strides made toward that end.

14.5.1 Bone Tissue Engineering

Osteoblasts contain high concentrations of polyP compared to other cell types in humans (Leyhausen et al. 1998). Further, polyP levels in primary human osteoblasts cultured in vitro are lowered by culture conditions known to stimulate osteoblast proliferation and differentiation, concomitant with an upregulation in exopolyphosphatase activity (Leyhausen et al. 1998). The phosphate polymers are present in the bone in vivo, as demonstrated by Omelon et al. (2009), who have confirmed their presence in resorption pits within mouse vertebral bodies (Omelon et al. 2009). Of relevance to their presence in the bone, the inhibitory effects of polyP on apatite crystal nucleation, growth, and solubility have been studied for more than half a century (Fleish and Neuman 1961; Francis 1969; McGaughey 1983). The effect on crystal growth occurs through a physicochemical process termed "crystal poisoning," whereby polyP are adsorbed to the growth sites of calcification nuclei and prevent further binding of ions. A number of studies have also demonstrated inhibitory effects of polyP on tissue calcification in vitro (Schroder et al. 2000; St-Pierre et al. 2010; Hoac et al. 2013) and in vivo (Fleisch et al. 1965; Irving et al. 1966; Schibler and Fleisch 1966).

Interestingly, it was also reported that embryonic chicken femur cultured *ex vivo* in media containing polyP exhibited significantly reduced mineral deposition at high doses in the bone but slightly increased mineralization at low doses (Fleisch et al. 1966). In support of this observation, Kawazoe et al. (2004) showed that the treatment of MC3T3-E1 preosteoblasts with polyP induced calcification in absence of another source of phosphate (Kawazoe et al. 2004). Further, more calcification was observed in cultures treated with an equivalent concentration of sodium orthophosphate or in typical calcification inducing conditions, such as in the presence of β-glycerophosphate. This stimulatory effect on mineralization was also observed by

others using a different osteoblastic cell line (Muller et al. 2011). These seemingly conflicting observations on the effects of exogenous polyP treatment may be attributed in part to the action of exopolyphosphatases, which lead to the release of orthophosphate ions that can participate in the mineralization process. It is probable that the ratio of polyP content-to-exopolyphosphatase activity helps dictate the inhibitory or promoting potential of condensed phosphates on mineralization, with the studies just discussed suggesting that the presence of other sources of cleavable phosphate may impact the balance. It also appears that the cation selected to form a salt with polyP plays a role in dictating the extent of mineral deposition by osteoblasts, with Ca^{2+} and Gd^{3+} providing improved responses compared to Na^{+} (Wang et al. 2013, 2016b). A number of studies have also described biological functions for polyP with potential implications for osteogenesis, notably through the modulation of FGF-2 (Kawazoe et al. 2008) and BMP-2 (Wang et al. 2013) signaling pathways. Similarly, polyP treatment of human mesenchymal stem cells and dental pulp cells stimulated osteoblastic differentiation (Kawazoe et al. 2008; Morimoto et al. 2010). It has also been shown to inhibit tartrate-resistant acid phosphatase and bone resorption in osteoclasts (Wang et al. 2013; Harada et al. 2013). Based on these results, it is not surprising that polyP injections in rat mandibular bone defects resulted in increased bone repair compared to controls (Hacchou et al. 2007).

These findings have served as a foundation for the development of a number of TERM strategies aimed at the delivery and retention of polyP within bone defects whereby normal repair processes are not sufficient to stimulate new tissue formation. One such approach entails the adsorption of polyP onto hydroxyapatite (IP-CHA), based on the ability of polyP to "poison" apatite (Morita et al. 2010). The authors report a rapid elution of the bioactive molecules from the scaffold, associated with enhanced early bone regeneration by 2 weeks after implantation in rabbit femurs at higher percentages of polyP (Fig. 14.3). PolyP-functionalized IP-CHA scaffolds also caused increased bone-implant contact 12 weeks after implantation in a granule format following tooth extraction in Beagle-Labrador dogs (Doi et al. 2014). In another study, the binding affinity of polyP for FGF-2 and its associated physical and functional stabilization (Shiba et al. 2003) were exploited to co-deliver the two bioactive molecules from IP-CHA scaffolds (Yuan et al. 2009). As expected from extensive in vitro data by the group, the combined treatment resulted in increased bone regeneration compared to the two individual treatments. These studies are of interest because they take advantage of the well-established benefits of HA as a scaffold for bone repair and exploit the binding affinity of polyP and apatite to enhance the biological response to the biomaterial.

Alternatively, Wu et al. (2015) devised a strategy to covalently link polyP to hyaluronic acid and generate a hydrogel that can be used to encapsulate and deliver cells to a bone defect (Wu et al. 2015). This approach, aimed at ensuring the constant stimulation of encapsulated preosteoblastic cells with the bioactive inorganic polymer, was shown to upregulate osteoblastic gene expression and result in increased alkaline phosphatase activity and calcium accumulation compared to conditions where the polyP was added to the culture medium or was free in the hydrogel. In a recent study, show that the use of sodium tripolyphosphate as a cross-linker

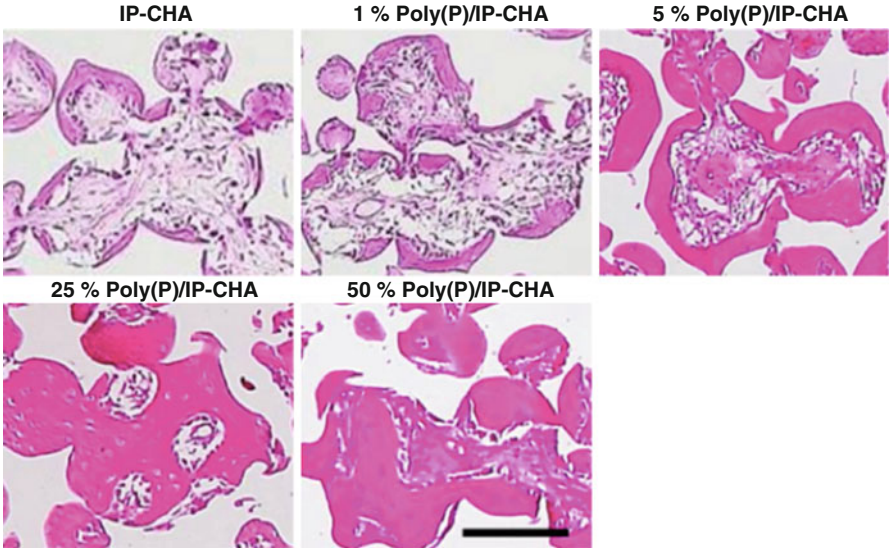

Fig. 14.3 Histological (hematoxylin and eosin) sections of bone tissue ingrowth within IP-CHA scaffolds alone or soaked in polyP solutions at different (w/w) percentage. Scale bar is 100 μm (Figure reproduced with permission from Morita et al. (2010))

in the fabrication of chitosan fibers by wet spinning was sufficient to enhance the differentiation of MG63 human osteoblastic cells. Others have delivered polyP to (Neufurth et al. 2014) or embedded it in (Wang et al. 2014) alginate/gelatin hydrogels encapsulating osteogenic cells and prepared by 3D bioprinting.

Taking a different approach, Müller et al. (2015) devised a methodology to produce amorphous nanoparticles of Ca^{2+}-polyP that can be delivered to osteogenic cells (Müller et al. 2015). These nanoparticles are characterized by an average size of 200 nm and stimulate mineral deposition when administered to SaOS-2 human osteosarcoma cells compared to larger Na^{+}-polyP particles, as well as nano-HA and β-TCP particles. Interestingly, the authors demonstrated a partial inhibition of the effects of the Ca^{2+}-polyP nanoparticles when clathrin-mediated endocytosis was prevented following treatment with trifluprom azine, suggesting a mechanism of internalization may be involved in mediating its biological effect (Müller et al. 2015b).

14.5.2 Articular Cartilage Regeneration

While very little is known as of yet on the biological roles polyP plays in cartilaginous tissues, the condensed phosphates have been identified in the hypertrophic zone of the growth plate cartilage (Omelon et al. 2009) and in the deep zone of the articular cartilage (unpublished data). Our group has recently demonstrated that polyP can be used to stimulate the accumulation of the major extracellular matrix (St. Pierre et al. 2012) components of the cartilage, collagen, and

glycosaminoglycans, by 3D high-density cultures of chondrocytes and by articular cartilage cultured *ex vivo* (St-Pierre et al. 2012). This effect was dose and chain length dependent with polyP chains of 45 orthophosphate units exhibiting an optimal response. Further, polyphosphate treatment had to be maintained throughout the culture period, owing to the susceptibility to exopolyphosphatases. Our group has also demonstrated that treatment of mineralizing tissue-engineered cartilage constructs with polyP prevents the deposition of a zone of the calcified cartilage (St-Pierre et al. 2010). The localization of polyP in the articular cartilage and its inhibitory role on cartilage calcification lead us to speculate as to a potential role in modulating the advancement of the tidemark delimiting the interface between the hyaline and calcified portions of the tissue. Of relevance, the development of osteoarthritis is associated with increased alkaline phosphatase activity, an enzyme with exopolyphosphatase activity and with increased rate of advancement of the tidemark (Burr 2004).

14.5.3 Tissue-Engineered Vascular Grafts

Tissue engineering has also shown promise in the development of vascular grafts to address the growing need for blood vessel reconstruction. However, the success of prosthetic grafts for the replacement of blood vessels smaller than 6 mm has been limited as a result of thrombogenicity (L'Heureux et al. 2007). A recent study aimed at addressing these issues has described the development of small tissue-engineered blood vessels composed of chitosan and alginate by extrusion into $CaCl_2$ (Neufurth et al. 2015). PolyP was incorporated into the extruded hydrogel to stimulate cell responses. The elastic modulus of the vessel was 475 kPa, and they survived 4 weeks of pulsatile flow. Importantly, the incorporation of polyP did not cause adverse secondary effects on clotting of human plasma. Based on other literature related to osteoblasts, polyP may have a positive effect on differentiation of mesenchymal stromal cells to endothelial cells, but this is yet to be shown.

14.5.4 Other Uses of Inorganic Polyphosphate in Tissue Engineering

PolyP has also been used to control infection and inflammation. For example, the topical application of polyP in a rat model of periodontal inflammation resulted in a decrease in the number of polymorphonuclear leukocytes. Increased collagen and FGF-2 levels in periodontal tissues treated with polyP also accompanied this effect. Of note, polyP treatment also caused the upregulation of tissue inhibitor of metalloproteinases (TIMPs), suggesting a modulation of the balance between catabolic and anabolic processes (Kasuyama et al. 2012). Topical administration of polyP to patients with periodontitis for an average of 19.6 weeks resulted in decreased levels of cytokine (IL-1β) and inflammation. Importantly it was also found to have no adverse effects and thus deemed safe for clinical use (Yamaoka et al. 2008).

These results are broad-reaching in that they suggest a potential benefit of the delivery of polyP to manage the consequences of inflammation. As many TERM applications involve the implantation of advanced materials in diseased tissues, inflammation is a factor that must be managed to provide an adequate environment for tissue repair and/or regeneration.

14.5.5 Inorganic Polyphosphate as a Delivery Vehicle

PolyP has been used in different ways to act as a delivery agent. l-tyrosine polyP (LTP-pDNA) nanoparticles were created as a nonviral vector for gene delivery (Ditto et al. 2013).

PolyP has recently been formed as dendrimers that could be utilized for the delivery of anabolic factors to targeted sites by incorporating growth factors or charged peptides such as Link-N (Caminade et al. 2011). The high charge density of polyP can promote efficient binding of biomolecules and release them either by time-dependent hydrolysis of polyP dendrimers at physiological pH or cleavage of PO_4 units from polyP chain by the resident cells of the tissue targeted by this delivery strategy. Nevertheless, the eventual success of this strategy still remains to be explored.

In another approach, ternary polymeric nanoparticles were prepared by ionic gelation of thiolated trimethyl chitosan and tripolyphosphate, and these were shown to be able to deliver siRNA to cells and to have systemic distribution after oral administration (Zhang et al. 2013).

These few examples highlight the possible role of polyP in creation of next generation of vehicles for the delivery of selected cargo. Exploration of the effects of polyP chain length and coadministration of polyanionic cross-linkers will also provide interesting opportunities for improved control of the kinetics of delivery.

14.6 Emerging Applications of Inorganic Polyphosphate in Tissue Engineering

As our understanding of the diverse effects of polyP expands, novel uses for this macromolecule are inevitable. Some of those on the horizon are reviewed next.

14.6.1 Modulating Cellular Utilization of Energy

Owing to their high-energy phosphoanhydride bonds, polyP has long been presumed to act as a source of energy for metabolic processes in mammalian cells. Pavlov et al. (2010) demonstrated a link between the metabolic state of astrocyte mitochondria and endogenous polyphosphate levels (Pavlov et al. 2010). The study also established that the mitochondrial polyP levels dictate ATP synthesis. Interestingly, from a tissue engineering perspective, it was shown that the administration of the

Ca^{2+}-polyP nanoparticles discussed previously to SaOS-2 cells leads to an increased number of mitochondria per cell (Müller et al. 2015a). This effect was accompanied by significant increases in both intracellular and extracellular ATP contents, thereby suggesting an approach to stimulate cellular energy levels. Such an effect of intracellular polyP delivery holds promise for a number of applications in tissue engineering, whereby the high energy requirements of cells must be met in order to sustain the anabolic processes required for rapid tissue regeneration. From the perspective of a recent push for the development of strategies for mitochondrial biogenesis in the treatment of a number of diseases associated with mitochondrial abnormalities (Whitaker et al. 2016; Wang et al. 2016c), the approach demonstrated in this work could also find broad applications in regenerative medicine.

14.6.2 Modulation of Blood Clotting

As with osteoblasts, platelet cellular bodies termed dense granules are characterized by high-polyP content (Ruiz et al. 2004). This observation has led to numerous studies to determine the roles played by these inorganic polymers in blood. It was notably demonstrated that polyP accumulating in platelet dense granules are released upon platelet activation and act as procoagulant and proinflammatory agents (Smith et al. 2006, 2010; Muller et al. 2009; Smith and Morrissey 2008). Based on these findings, a number of groups have recently developed nanoparticle systems containing polyP to accelerate blood clotting with the aim of controlling hemorrhage (Donovan et al. 2014; Szymusiak et al. 2016; Kudela et al. 2015). The inorganic polymer is a particularly interesting candidate for the management of bleeding events as it accelerates clotting but acts as a poor initiator of coagulation processes and may be suitable for intravenous administration. Further functionalization of the nanoparticle carriers would then provide an opportunity for targeted delivery (Kudela et al. 2015). A recent study by Momeni et al. (2016) demonstrated the use of Na^{+} polyP to create a radiopaque liquid embolic formulation and successfully achieve the occlusion of an auricular artery in a rabbit model without cell death. This was achieved by substituting Na^{2+} with Ca^{2+}, Sr^{2+}, or Ba^{2+} to generate different coacervates with varying viscosities and opacities. The formulations were further optimized to achieve the final coacervate with desired viscosity for injectability, opacity for visualization during the embolic procedure and with no cytotoxic effects (Momeni et al. 2016). Though a pilot study, this application might find use in tissue implantation in vivo for controlled occlusion-reperfusion strategy.

Other studies have aimed at preventing the procoagulant and proinflammatory effects of endogenous polyP. Smith et al. (2012) and Zhu et al. (2015) investigated the potential of a vast array of polyP inhibitors to prevent thrombosis and inflammation in vitro and in vivo (Smith et al. 2012; Zhu et al. 2015). These studies highlight the fact that the inhibition of endogenous polyP effects may prove beneficial in some conditions, in others may be deleterious. These studies also stress the importance of gaining increased insight into the roles of polyP in tissue homeostasis and the development of pathologies.

14.6.3 Inorganic Polyphosphate in Skin Tissue Engineering and Wound Healing

PolyP may also have uses in skin- and wound-healing tissue engineering. Müller et al. (2015b) developed Ca^{2+}-polyP amorphous nanoparticles which incorporated retinol for the co-delivery of the two bioactive molecules to be used for dermal tissue engineering applications (Müller et al. 2015b). This study builds on previously described work to exploit the physicochemical properties and degradability of polyP for the development of drug delivery carriers but also notes that the careful selection of cargo to be co-delivered with polyP can lead to synergistic biological effects. It was hypothesized that the demonstrated effects of polyP on collagen expression (Hacchou et al. 2007) and their antibacterial properties (Florin and Thelestam 1984) might be beneficial as a therapeutic approach to limit changes associated with skin aging. Retinol was included as it has a well-established role in maintaining skin health (Mukherjee et al. 2006). The study demonstrated that the co-delivery of Ca^{2+}-polyP nanoparticles and retinol at concentrations lower than those demonstrating a biological effect for each compound alone leads to synergistic effects on MC3T3 cells and upregulation of the expression of collagen types I, II, and III but not V. This response appeared to be regulated in part by clathrin-mediated uptake of the nanospheres. In an another study by Müller et al. (2015c, d), the authors incorporated the Ca^{2+}-polyP/retinol nanoparticles within the fibers of poly (lactic acid) electrospun membranes to generate a wound dressing. MC3T3-E1 cells incubated on these dressings were found to exhibit increased FABP4, leptin, and leptin receptor gene expression compared to controls. The authors speculated that FABP4 could bind to the retinoic acid and translocate to the nucleus, whereby leptin and leptin receptor expression is stimulated with potential reepithelialization effects (Müller et al. 2015c, d). Such an approach maybe of benefit for the delivery of expensive molecules, including growth factors, as the therapeutic dose could be substantially decreased as a previous study highlighted the increased half-life of bFGF upon interaction with polyP (Shiba et al. 2003).

14.7 Concluding Remarks

In recent years, we have witnessed a shift in the use of polyP in TERM applications from its utilization as a building block for the development of biomaterials with interesting physicochemical properties to its administration as a bioactive molecule that enables the modulation of cellular and tissue responses. There has been an increase in the number of studies highlighting the benefits of harnessing and/or modulating polyP bioactivity for tissue engineering purposes (summarized in Fig. 14.4). As such, the field has only tapped a small fraction of the broad range of biological effects of polyP. In fact, while polyP have been described as ubiquitous in mammalian cells and their levels regulated in response to stimuli, the specific functions fulfilled by these inorganic polymers in tissue homeostasis remain largely unresolved in most biological systems. In much the same way, potential implications in pathological pathways mostly remain to be explored. Improved understanding of the tissue-specific roles of polyP is bound to provide new opportunities for

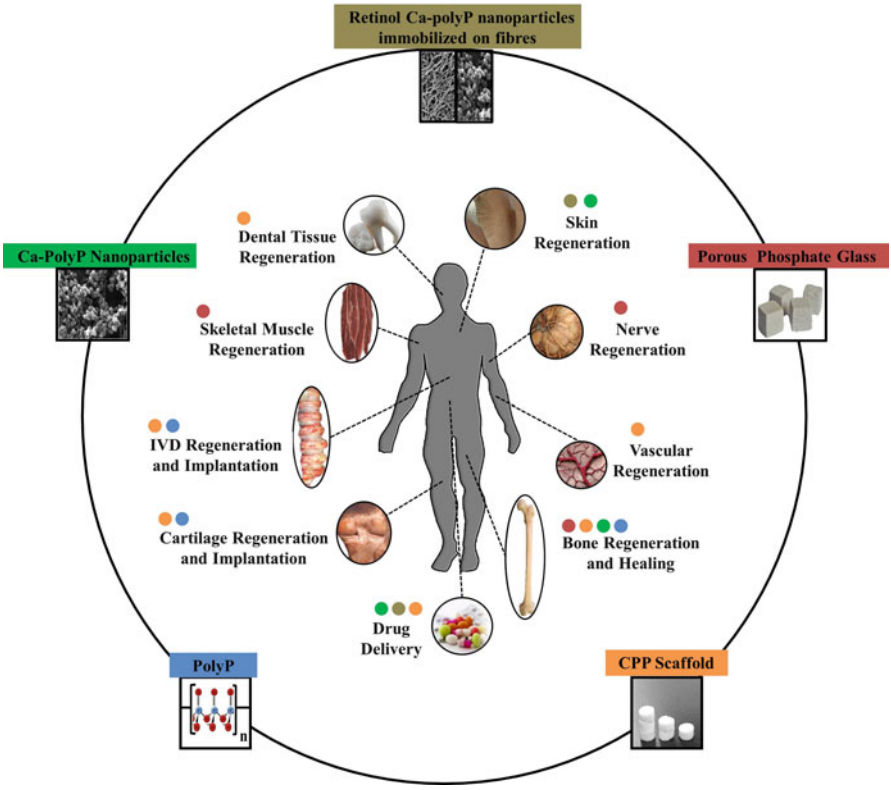

Fig. 14.4 Schematic depicting different formulations of polyP used for tissue engineering and regenerative medicine (*TERM*) applications. *Colored dots* indicate use of the different formulations of polyP (*colored boxes*) used for that particular activity. Ca-polyP and nanofiber pictures were reproduced with permission from (Müller et al. (2015c); Urch et al. (2009). Picture of porous phosphate glass was a kind contribution by Prof. Dr. Delia Brauer, Otto Schott Institute of Materials Research, Friedrich-Schiller-Universität Jena, Germany

TERM approaches. The fact that small concentrations of the phosphate polymer are generally required for biological activity and that it is readily degraded to orthophosphate in the body are advantageous features for TERM applications. However, translation of strategies for the delivery of polyP would greatly benefit from the development of strategies to reliably monitor the pharmacokinetics of polyP in the body without affecting bioactivity. While important advances are being achieved with these materials, it is also important to stress the need for caution in employing polyP for TERM applications. PolyP has been shown to exhibit opposite effects with different cell types for a number of biological effects including proliferation and mineralization. Further, controversy still exists as to their effects in a number of biological systems. As such, it will be important to monitor and understand the potential effects of polyP on all tissues where they are deployed as therapeutic agents. Continued efforts to gain insight into the tissue-specific pathways modulated by polyP will facilitate the translation of TERM strategies incorporating polyP

to the clinic. Continued efforts to demonstrate the benefits of combining polyP with more traditional bioactive agents for improved tissue response will also contribute to making this molecule more widely used in TERM applications. Clearly the studies to date only describe the tip of the iceberg in regards to the potential of polyP for TERM applications and that promising new applications for polyP in improving health very likely will continue to develop.

Acknowledgments This work was supported by CIHR grant number CIHR-MOP12611 to RK. RG was supported by postdoctoral fellowships from The Toronto Musculoskeletal Centre (TMC), University of Toronto; The Arthritis Society (TAS), Canada; and the National Science and Educational Research Council of Canada (NSERC). We thank Dr. Justin Parreno and Prof. Dr. Delia Brauer, Otto Schott Institute of Materials Research, Friedrich-Schiller-Universität Jena, Germany for their help in generating Fig. 14.4.

References

Abou Neel EA, Knowles JC (2008) Physical and biocompatibility studies of novel titanium dioxide doped phosphate-based glasses for bone tissue engineering applications. J Mater Sci Mater Med 19(1):377–386. doi:10.1007/s10856-007-3079-5

Abou Neel EA, Pickup DM, Valappil SP et al (2009) Bioactive functional materials: a perspective on phosphate-based glasses. J Mater Chem 19(6):690–701. doi:10.1039/B810675D

Al Qaysi M, Walters NJ, Foroutan F et al (2015) Strontium- and calcium-containing, titanium-stabilised phosphate-based glasses with prolonged degradation for orthopaedic tissue engineering. J Biomater Appl 30(3):300–310. doi:10.1177/0885328215588898

Baino F, Novajra G, Vitale-Brovarone C (2015) Bioceramics and scaffolds: a winning combination for tissue engineering. Front Bioeng Biotechnol 3:202. doi:10.3389/fbioe.2015.00202

Bitar M, Salih V, Mudera V et al (2004) Soluble phosphate glasses: in vitro studies using human cells of hard and soft tissue origin. Biomaterials 25(12):2283–2292

Burr DB (2004) Anatomy and physiology of the mineralized tissues: role in the pathogenesis of osteoarthrosis. Osteoarthr Cartil/OARS, Osteoarthritis Res Soc 12(Suppl A):S20–S30

Caminade A-M, Turrin C-O, Laurent R, Ouali A, Delavaux-Nicot B (2011) Dendrimers: towards catalytic, material and biomedical uses. Wiley, Hoboken

Cassidy JD, Carroll LJ, Cote P (1998) The Saskatchewan health and back pain survey. The prevalence of low back pain and related disability in Saskatchewan adults. Spine 23(17):1860–1866; discussion 1867

Chen YW, Feng T, Shi GQ et al (2008a) Interaction of endothelial cells with biodegradable strontium-doped calcium polyphosphate for bone tissue engineering. Appl Surf Sci 255(2):331–335, http://dx.doi.org/10.1016/j.apsusc.2008.06.154

Chen YW, Shi GQ, Ding YL et al (2008b) In vitro study on the influence of strontium-doped calcium polyphosphate on the angiogenesis-related behaviors of HUVECs. J Mater Sci Mater Med 19(7):2655–2662. doi:10.1007/s10856-007-3350-9

Chen XR, Bai J, Yuan SJ et al (2015) Calcium phosphate nanoparticles are associated with inorganic phosphate-induced osteogenic differentiation of rat bone marrow stromal cells. Chem Biol Interact 238:111–117. doi:10.1016/j.cbi.2015.06.027

Chen L, Song W, Markel DC et al (2016) Flow perfusion culture of MC3T3-E1 osteogenic cells on gradient calcium polyphosphate scaffolds with different pore sizes. J Biomater Appl 30(7):908–918. doi:10.1177/0885328215608335

Delahaye F, Montagne L, Palavit G et al (1998) Acid dissolution of sodium–calcium metaphosphate glasses. J Non-Cryst Solids 242(1):25–32, http://dx.doi.org/10.1016/S0022-3093(98)00784-4

Ditto AJ, Reho JJ, Shah KN et al (2013) In vivo gene delivery with L-tyrosine polyphosphate nanoparticles. Mol Pharm 10(5):1836–1844. doi:10.1021/mp300623a

Doi K, Kubo T, Takeshita R et al (2014) Inorganic polyphosphate adsorbed onto hydroxyapatite for guided bone regeneration: an animal study. Dent Mater J 33(2):179–186
Donovan AJ, Kalkowski J, Smith SA et al (2014) Size-controlled synthesis of granular polyphosphate nanoparticles at physiologic salt concentrations for blood clotting. Biomacromolecules 15(11):3976–3984. doi:10.1021/bm501046t
EI Sayegh TY, Pilliar RM, McCulloch CA (2002) Attachment, spreading, and matrix formation by human gingival fibroblasts on porous-structured titanium alloy and calcium polyphosphate substrates. J Biomed Mater Res 61(3):482–492. doi:10.1002/jbm.10217
Fleisch H, Schibler D, Maerki J, Frossard I (1965) Inhibition of aortic calcification by means of pyrophosphate and polyphosphates. Nature 207(5003):1300–1301
Fleisch H, Straumann F, Schenk R et al (1966) Effect of condensed phosphates on calcification of chick embryo femurs in tissue culture. Am J Physiol 211(3):821–825
Fleish H, Neuman WF (1961) Mechanisms of calcification: role of collagen, polyphosphates, and phosphatase. Am J Physiol 200:1296–1300
Florin I, Thelestam M (1984) Polyphosphate-mediated protection from cellular intoxication with Clostridium difficile toxin B. Biochim Biophys Acta 805(2):131–136
Francis MD (1969) The inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates. Calcif Tissue Res 3(2):151–162
Fu WL, Xiang Z, Huang FG et al (2015) Coculture of peripheral blood-derived mesenchymal stem cells and endothelial progenitor cells on strontium-doped calcium polyphosphate scaffolds to generate vascularized engineered bone. Tissue Eng Part A 21(5–6):948–959. doi:10.1089/ten.TEA.2014.0267
Griffin JB, Davidian NM, Penniall R (1965) Studies of phosphorus metabolism by isolated nuclei. VII. Identification of polyphosphate as a product. J Biol Chem 240(11):4427–4434
Griffith EJ (1995) Phosphate fibers. Topics in applied chemistry. Plenum Publishing Corporation, New York
Grynpas MD, Pilliar RM, Kandel RA et al (2002) Porous calcium polyphosphate scaffolds for bone substitute applications in vivo studies. Biomaterials 23(9):2063–2070
Gu Z, Wang H, Li L et al (2012) Cell-mediated degradation of strontium-doped calcium polyphosphate scaffold for bone tissue engineering. Biomed Mater 7(6):065007. doi:10.1088/1748-6041/7/6/065007
Gu Z, Zhang X, Li L et al (2013) Acceleration of segmental bone regeneration in a rabbit model by strontium-doped calcium polyphosphate scaffold through stimulating VEGF and bFGF secretion from osteoblasts. Mater Sci Eng C Mater Biol Appl 33(1):274–281. doi:10.1016/j.msec.2012.08.040
Hacchou Y, Uematsu T, Ueda O et al (2007) Inorganic polyphosphate: a possible stimulant of bone formation. J Dent Res 86(9):893–897
Hamilton DJ, Seguin CA, Wang J et al (2006) Formation of a nucleus pulposus-cartilage endplate construct in vitro. Biomaterials 27(3):397–405. doi:10.1016/j.biomaterials.2005.07.007
Harada K, Itoh H, Kawazoe Y et al (2013) Polyphosphate-mediated inhibition of tartrate-resistant acid phosphatase and suppression of bone resorption of osteoclasts. PLoS One 8(11):e78612. doi:10.1371/journal.pone.0078612
Hench LL, Wilson J (1984) Surface-active biomaterials. Science 226(4675):630–636
Hench & Jones (2015) Front Bioeng Biotechnol. Bioactive Glasses: Frontiers and Challenges. eCollection 30(3):194. doi: 10.3389/fbioe.2015.00194
Hoac B, Kiffer-Moreira T, Millan JL et al (2013) Polyphosphates inhibit extracellular matrix mineralization in MC3T3-E1 osteoblast cultures. Bone 53(2):478–486. doi:10.1016/j.bone.2013.01.020
Hu Y, Shanjani Y, Toyserkani E, Grynpas M et al (2014) Porous calcium polyphosphate bone substitutes: additive manufacturing versus conventional gravity sinter processing effect on structure and mechanical properties. J biomd Mater Res Appl Biomater 102(2):274–83.
Irving JT, Schibler D, Fleisch H (1966) Effect of condensed phosphates on vitamin D-induced aortic calcification in rats. Proc Soc Exp Biol Med 122(3):852–856
Kandel RA, Grynpas M, Pilliar R et al (2006) Repair of osteochondral defects with biphasic cartilage-calcium polyphosphate constructs in a sheep model. Biomaterials 27(22):4120–4131. doi:10.1016/j.biomaterials.2006.03.005

Kang P, Xie X, Tan Z et al (2015) Repairing defect and preventing collapse of femoral head in a steroid-induced osteonecrotic of femoral head animal model using strontium-doped calcium polyphosphate combined BM-MNCs. J Mater Sci Mater Med 26(2):80. doi:10.1007/s10856-015-5402-x

Kasuyama K, Tomofuji T, Ekuni D et al (2012) Effects of topical application of inorganic polyphosphate on tissue remodeling in rat inflamed gingiva. J Periodontal Res 47(2):159–164. doi:10.1111/j.1600-0765.2011.01414.x

Kawazoe Y, Shiba T, Nakamura R et al (2004) Induction of calcification in MC3T3-E1 cells by inorganic polyphosphate. J Dent Res 83(8):613–618

Kawazoe Y, Katoh S, Onodera Y et al (2008) Activation of the FGF signaling pathway and subsequent induction of mesenchymal stem cell differentiation by inorganic polyphosphate. Int J Biol Sci 4(1):37–47

Kim YP, Lee GS, Kim JW et al (2015) Phosphate glass fibres promote neurite outgrowth and early regeneration in a peripheral nerve injury model. J Tissue Eng Regen Med 9(3):236–246. doi:10.1002/term.1626

Kudela D, Smith SA, May-Masnou A et al (2015) Clotting activity of polyphosphate-functionalized silica nanoparticles. Angew Chem Int Ed Engl 54(13):4018–4022. doi:10.1002/anie.201409639

Kumble KD, Kornberg A (1995) Inorganic polyphosphate in mammalian cells and tissues. J Biol Chem 270(11):5818–5822

Lakhkar NJ, R MD, Kim HW et al (2015) Titanium phosphate glass microcarriers induce enhanced osteogenic cell proliferation and human mesenchymal stem cell protein expression. J Tiss Eng 6:2041731415617741. doi: 10.1177/2041731415617741

Lee WD, Hurtig MB, Pilliar RM et al (2015) Engineering of hyaline cartilage with a calcified zone using bone marrow stromal cells. Osteoarthr Cartil 23(8):1307–1315. doi:10.1016/j.joca.2015.04.010

Leyhausen G, Lorenz B, Zhu H et al (1998) Inorganic polyphosphate in human osteoblast-like cells. J Bone Miner Res 13(5):803–812. doi:10.1359/jbmr.1998.13.5.803

L'Heureux N, Dusserre N, Marini A et al (2007) Technology insight: the evolution of tissue-engineered vascular grafts – from research to clinical practice. Nat Clin Pract Cardiovasc Med 4(7):389–395. doi:10.1038/ncpcardio0930

Liu F, Zhang X, Yu X et al (2011) In vitro study in stimulating the secretion of angiogenic growth factors of strontium-doped calcium polyphosphate for bone tissue engineering. J Mater Sci Mater Med 22(3):683–692. doi:10.1007/s10856-011-4247-1

Lorenz B, Munkner J, Oliveira MP et al (1997) Changes in metabolism of inorganic polyphosphate in rat tissues and human cells during development and apoptosis. Biochim Biophys Acta 1335(1–2):51–60

Lynn WS, Brown RH (1963) Synthesis of polyphosphate by rat liver mitochondria. Biochem Biophys Res Commun 11:367–371

McGaughey C (1983) Binding of polyphosphates and phosphonates to hydroxyapatite, subsequent hydrolysis, phosphate exchange and effects on demineralization, mineralization and microcrystal aggregation. Caries Res 17(3):229–241

Momeni A, Valliant EM, Brennan-Pierce EP et al (2016) Developing an in situ forming polyphosphate coacervate as a new liquid embolic agent: from experimental design to pilot animal study. Acta Biomater 32:286–297. doi:10.1016/j.actbio.2015.12.012

Morimoto D, Tomita T, Kuroda S et al (2010) Inorganic polyphosphate differentiates human mesenchymal stem cells into osteoblastic cells. J Bone Miner Metab 28(4):418–423. doi:10.1007/s00774-010-0157-4

Morita K, Doi K, Kubo T et al (2010) Enhanced initial bone regeneration with inorganic polyphosphate-adsorbed hydroxyapatite. Acta Biomater 6(7):2808–2815. doi:10.1016/j.actbio.2009.12.055

Mukherjee S, Date A, Patravale V et al (2006) Retinoids in the treatment of skin aging: an overview of clinical efficacy and safety. Clin Interv Aging 1(4):327–348

Muller F, Mutch NJ, Schenk WA et al (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell 139(6):1143–1156. doi:10.1016/j.cell.2009.11.001

Muller WE, Wang X, Diehl-Seifert B et al (2011) Inorganic polymeric phosphate/polyphosphate as an inducer of alkaline phosphatase and a modulator of intracellular Ca^{2+} level in osteoblasts (SaOS-2 cells) in vitro. Acta Biomater 7(6):2661–2671. doi:10.1016/j.actbio.2011.03.007

Müller WE, Tolba E, Feng Q et al (2015a) Amorphous Ca(2)(+) polyphosphate nanoparticles regulate the ATP level in bone-like SaOS-2 cells. J Cell Sci 128(11):2202–2207. doi:10.1242/jcs.170605

Müller WE, Tolba E, Schroder HC et al (2015b) Retinol encapsulated into amorphous Ca(2+) polyphosphate nanospheres acts synergistically in MC3T3-E1 cells. Eur J Pharm Biopharm 93:214–223. doi:10.1016/j.ejpb.2015.04.005

Müller WEG, Tolba E, Dorweiler B et al (2015c) Electrospun bioactive mats enriched with Ca-polyphosphate/retinol nanospheres as potential wound dressing. Biochem Biophys Rep 3:150–160, http://dx.doi.org/10.1016/j.bbrep.2015.08.007

Müller WEG, Tolba E, Schröder HC et al (2015d) A new polyphosphate calcium material with morphogenetic activity. Mater Lett 148:163–166, http://dx.doi.org/10.1016/j.matlet.2015.02.070

Nelson SR, Wolford LM, Lagow RJ et al (1993) Evaluation of new high-performance calcium polyphosphate bioceramics as bone graft materials. J Oral Maxillofac Surg 51(12):1363–1371

Neufurth M, Wang X, Schroder HC et al (2014) Engineering a morphogenetically active hydrogel for bioprinting of bioartificial tissue derived from human osteoblast-like SaOS-2 cells. Biomaterials 35(31):8810–8819. doi:10.1016/j.biomaterials.2014.07.002

Neufurth M, Wang X, Tolba E et al (2015) Modular small diameter vascular grafts with bioactive functionalities. PLoS One 10(7):e0133632. doi:10.1371/journal.pone.0133632

Omelon S, Georgiou J, Henneman ZJ et al (2009) Control of vertebrate skeletal mineralization by polyphosphates. PLoS One 4(5):e5634. doi:10.1371/journal.pone.0005634

Pavlov E, Aschar-Sobbi R, Campanella M et al (2010) Inorganic polyphosphate and energy metabolism in mammalian cells. J Biol Chem 285(13):9420–9428. doi:10.1074/jbc.M109.013011

Petrera M, De Croos JN, Iu J et al (2013) Supplementation with platelet-rich plasma improves the in vitro formation of tissue-engineered cartilage with enhanced mechanical properties. Arthroscopy 29(10):1685–1692. doi:10.1016/j.arthro.2013.07.259

Petrone C, Hall G, Langman M et al (2008) Compaction strategies for modifying the drug delivery capabilities of gelled calcium polyphosphate matrices. Acta Biomater 4(2):403–413. doi:10.1016/j.actbio.2007.09.007

Pilliar RM, Filiaggi MJ, Wells JD et al (2001) Porous calcium polyphosphate scaffolds for bone substitute applications – in vitro characterization. Biomaterials 22(9):963–972

Pilliar RM, Kandel RA, Grynpas MD, Hu Y (2013) Porous calcium polyphosphate as load-bearing bone substitutes: in vivo study. J Biomed Mater Res B Appl Biomater 101(1):1–8.

Pilliar RM, Kandel RA, Grynpas MD et al (2016) Calcium polyphosphate particulates for bone void filler applications. J Biomed Mater Res B Appl Biomater. doi:10.1002/jbm.b.33623

Pisoni RL, Lindley ER (1992) Incorporation of [32P]orthophosphate into long chains of inorganic polyphosphate within lysosomes of human fibroblasts. J Biol Chem 267(6):3626–3631

Ren WP, Song W, Esquivel AO et al (2014) Effect of erythromycin-doped calcium polyphosphate scaffold composite in a mouse pouch infection model. J Biomed Mater Res B Appl Biomater 102(6):1140–1147. doi:10.1002/jbm.b.33095

Ruiz FA, Lea CR, Oldfield E et al (2004) Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. J Biol Chem 279(43):44250–44257. doi:10.1074/jbc.M406261200

Schibler D, Fleisch H (1966) Inhibition of skin calcification (calciphylaxis) by polyphosphates. Experientia 22(6):367–369

Schofield SC, Berno B, Langman M et al (2006) Gelled calcium polyphosphate matrices delay antibiotic release. J Dent Res 85(7):643–647

Schroder HC, Kurz L, Muller WE, Lorenz B (2000) Polyphosphate in bone. Biochemistry (Moscow) 65(3):296–303

Seguin CA, Grynpas MD, Pilliar RM et al (2004) Tissue engineered nucleus pulposus tissue formed on a porous calcium polyphosphate substrate. Spine 29(12):1299–1306; discussion 1306–1307

Shah R, Sinanan AC, Knowles JC et al (2005) Craniofacial muscle engineering using a 3-dimensional phosphate glass fibre construct. Biomaterials 26(13):1497–1505. doi:10.1016/j.biomaterials.2004.04.049

Shah R, Ready D, Knowles JC et al (2014) Sequential identification of a degradable phosphate glass scaffold for skeletal muscle regeneration. J Tissue Eng Regen Med 8(10):801–810. doi:10.1002/term.1581

Shanjani Y, Hu Y, Toyserkani E, Grynpas M et al (2013) Solid freeform fabrication of porous calcium polyphosphate structures for bone substitute applications: in vivo studies. J Biomed Mater Res B Appl Biomater 101(6):972–980. doi:10.1002/jbm.b.32905

Shiba T, Nishimura D, Kawazoe Y et al (2003) Modulation of mitogenic activity of fibroblast growth factors by inorganic polyphosphate. J Biol Chem 278(29):26788–26792. doi:10.1074/jbc.M303468200

Smith SA, Morrissey JH (2008) Polyphosphate as a general procoagulant agent. J Thromb Haemost 6(10):1750–1756. doi:10.1111/j.1538-7836.2008.03104.x

Smith SA, Mutch NJ, Baskar D et al (2006) Polyphosphate modulates blood coagulation and fibrinolysis. Proc Natl Acad Sci U S A 103(4):903–908. doi:10.1073/pnas.0507195103

Smith SA, Choi SH, Davis-Harrison R et al (2010) Polyphosphate exerts differential effects on blood clotting, depending on polymer size. Blood 116(20):4353–4359. doi:10.1182/blood-2010-01-266791

Smith SA, Choi SH, Collins JN et al (2012) Inhibition of polyphosphate as a novel strategy for preventing thrombosis and inflammation. Blood 120(26):5103–5110. doi:10.1182/blood-2012-07-444935

Song W, Tian M, Chen F et al (2009) The study on the degradation and mineralization mechanism of ion-doped calcium polyphosphate in vitro. J Biomed Mater Res B Appl Biomater 89(2):430–438. doi:10.1002/jbm.b.31230

Song W, Ren W, Wan C et al (2011) A novel strontium-doped calcium polyphosphate/erythromycin/poly(vinyl alcohol) composite for bone tissue engineering. J Biomed Mater Res A 98(3):359–371. doi:10.1002/jbm.a.33127

St- Pierre JP (2011) The role of inorganic polyphosphates in the formation of bioengineered cartilage incorporating a zone of calcified cartilage in vitro. University of Toronto Thesis Repository. http://hdl.handle.net/1807/33860:268

St-Pierre JP, Pilliar RM, Grynpas MD et al (2010) Calcification of cartilage formed in vitro on calcium polyphosphate bone substitutes is regulated by inorganic polyphosphate. Acta Biomater 6(8):3302–3309. doi:10.1016/j.actbio.2010.02.033

St-Pierre JP, Wang Q, Li SQ et al (2012) Inorganic polyphosphate stimulates cartilage tissue formation. Tissue Eng Part A 18(11–12):1282–1292. doi:10.1089/ten.TEA.2011.0356

Szymusiak M, Donovan AJ, Smith SA et al (2016) Colloidal confinement of polyphosphate on gold nanoparticles robustly activates the contact pathway of blood coagulation. Bioconjug Chem 27(1):102–109. doi:10.1021/acs.bioconjchem.5b00524

Urch H, Vallet-Regi M, Ruiz L, Gonzalez-Calbet JM, Epple M (2009) Calcium phosphate nanoparticles with adjustable dispersability and crystallinity. Journal of Materials Chemistry 19 (15):2166–2171. doi:10.1039/B810026H

Waldman SD, Grynpas MD, Pilliar RM et al (2002) Characterization of cartilagenous tissue formed on calcium polyphosphate substrates in vitro. J Biomed Mater Res 62(3):323–330. doi:10.1002/jbm.10235

Wang FM, Qiu K, Hu T et al (2006) Biodegradable porous calcium polyphosphate scaffolds for the three-dimensional culture of dental pulp cells. Int Endod J 39(6):477–483. doi:10.1111/j.1365-2591.2006.01114.x

Wang X, Schroder HC, Diehl-Seifert B et al (2013) Dual effect of inorganic polymeric phosphate/polyphosphate on osteoblasts and osteoclasts in vitro. J Tissue Eng Reg Med 7(10):767–776. doi:10.1002/term.1465

Wang X, Tolba E, Schroder HC et al (2014) Effect of bioglass on growth and biomineralization of SaOS-2 cells in hydrogel after 3D cell bioprinting. PLoS One 9(11):e112497. doi:10.1371/journal.pone.0112497

Wang X, Huang J, Wang K et al (2015) The morphogenetically active polymer, inorganic polyphosphate complexed with GdCl, as an inducer of hydroxyapatite formation in vitro. Biochem Pharmacol. doi:10.1016/j.bcp.2015.12.011

Wang X, Gu Z, Jiang B et al (2016a) Surface modification of strontium-doped porous bioactive ceramic scaffolds via poly(DOPA) coating and immobilizing silk fibroin for excellent angiogenic and osteogenic properties. Biomater Sci. doi:10.1039/c5bm00482a

Wang X, Huang J, Wang K et al (2016b) The morphogenetically active polymer, inorganic polyphosphate complexed with GdCl3, as an inducer of hydroxyapatite formation in vitro. Biochem Pharmacol 102:97–106. doi:10.1016/j.bcp.2015.12.011

Wang X, Schroder HC, Muller WE (2016c) Polyphosphate as a metabolic fuel in Metazoa: a foundational breakthrough invention for biomedical applications. Biotechnol J 11(1):11–30. doi:10.1002/biot.201500168

Whitaker RM, Corum D, Beeson CC et al (2016) Mitochondrial biogenesis as a pharmacological target: a new approach to acute and chronic diseases. Annu Rev Pharmacol Toxicol 56:229–249. doi:10.1146/annurev-pharmtox-010715-103155

Wu AT, Aoki T, Sakoda M et al (2015) Enhancing osteogenic differentiation of MC3T3-E1 cells by immobilizing inorganic polyphosphate onto hyaluronic acid hydrogel. Biomacromolecules 16(1):166–173. doi:10.1021/bm501356c

Xie H, Wang Q, Ye Q et al (2012) Application of K/Sr co-doped calcium polyphosphate bioceramic as scaffolds for bone substitutes. J Mater Sci Mater Med 23(4):1033–1044. doi:10.1007/s10856-012-4556-z

Yamaoka M, Uematsu T, Shiba T et al (2008) Effect of inorganic polyphosphate in periodontitis in the elderly. Gerodontology 25(1):10–17. doi:10.1111/j.1741-2358.2007.00185.x

Yuan Q, Kubo T, Doi K et al (2009) Effect of combined application of bFGF and inorganic polyphosphate on bioactivities of osteoblasts and initial bone regeneration. Acta Biomater 5(5):1716–1724. doi:10.1016/j.actbio.2009.01.034

Zhang J, He C, Tang C, Yin C (2013) Ternary polymeric nanoparticles for oral siRNA delivery. Pharm Res 30(5):1228–1239. doi:10.1007/s11095-012-0961-8

Zheng K, Wu Z, Wei J et al (2015) Preparation and characterization of fibrous chitosan-glued phosphate glass fiber scaffolds for bone regeneration. J Mater Sci Mater Med 26(8):224. doi:10.1007/s10856-015-5554-8

Zhou L, Hu CJ, Xu GP et al (2012) In vitro and in vivo study of calcium polyphosphate fiber/calcium phosphate cement/micromorselized bone composite for bone defect repair. J Biomed Mater Res B Appl Biomater 100(5):1190–1197. doi:10.1002/jbm.b.32644

Zhu S, Travers RJ, Morrissey JH et al (2015) FXIa and platelet polyphosphate as therapeutic targets during human blood clotting on collagen/tissue factor surfaces under flow. Blood 126(12):1494–1502. doi:10.1182/blood-2015-04-641472

Conclusion: Evolution of the Polyphosphate Role in Eukaryotes

Phosphorus, a vital cell element, is a part of nucleic acids, ATP and other nucleoside phosphates, phospholipids, phosphorylated proteins, and carbohydrates. Condensed phosphates were proposed to be formed under geochemical processes in primitive Earth and to be included in the primordial metabolism as an energy source molecule (Miller and Parris 1964; Beck and Orgel 1965; Osterberg and Orgel 1972). The modern hypothesis modeling life origin suggests the participation of inorganic phosphorus compounds in the early stages of chemical evolution (Pasek 2008; Holm and Baltscheffsky 2011; Norris et al. 2014; Barge et al. 2015).

The hypothesis that abiogenic polyphosphate formed as a result of geothermal activity could be used in ancient transphosphorylation processes is still under discussion.

The idea that the primary functions of polyP were phosphorus storage and energy molecule is based on the two properties of these biopolymers:

- PolyP is an osmotically neutral form of phosphorus.
- The energy of phosphoanhydride bonds between phosphate residues in PolyP is close to the energy of terminal phosphoester bond in ATP.

These functions of polyP are important in prokaryotes and conserved in the cells of lower eukaryote, especially in fungi. However, in lower eukaryotes the role of polyP in second messenger metabolism, stress response, and enzyme activities regulation is also highly significant. The cells of the higher eukaryotes are lesser dependent on the environment, and the accumulation of large amounts of polyP as a phosphorus reserve could have been lost during the evolutionary process.

The ability of polyP to form complexes with cationic compounds directly and with negatively charged compounds via metal cations was probably demanded at earliest stages of evolution. PolyP forms specific complexes with many biopolymers, including poly-β-hydroxybutyrate, RNA, and proteins. This ability of PolyP allows them to participate in membrane transport, regulation of gene expression, and enzyme activities. These properties are the basis of multiple polyP functions in the cells of higher eukaryotes (Fig. 1).

One of pathways of phosphorus biomineralization had conserved during the evolution from prokaryotes to the higher eukaryotes. There is a similarity between

T. Kulakovskaya et al. (eds.), *Inorganic Polyphosphates in Eukaryotic Cells*,
DOI 10.1007/978-3-319-41073-9

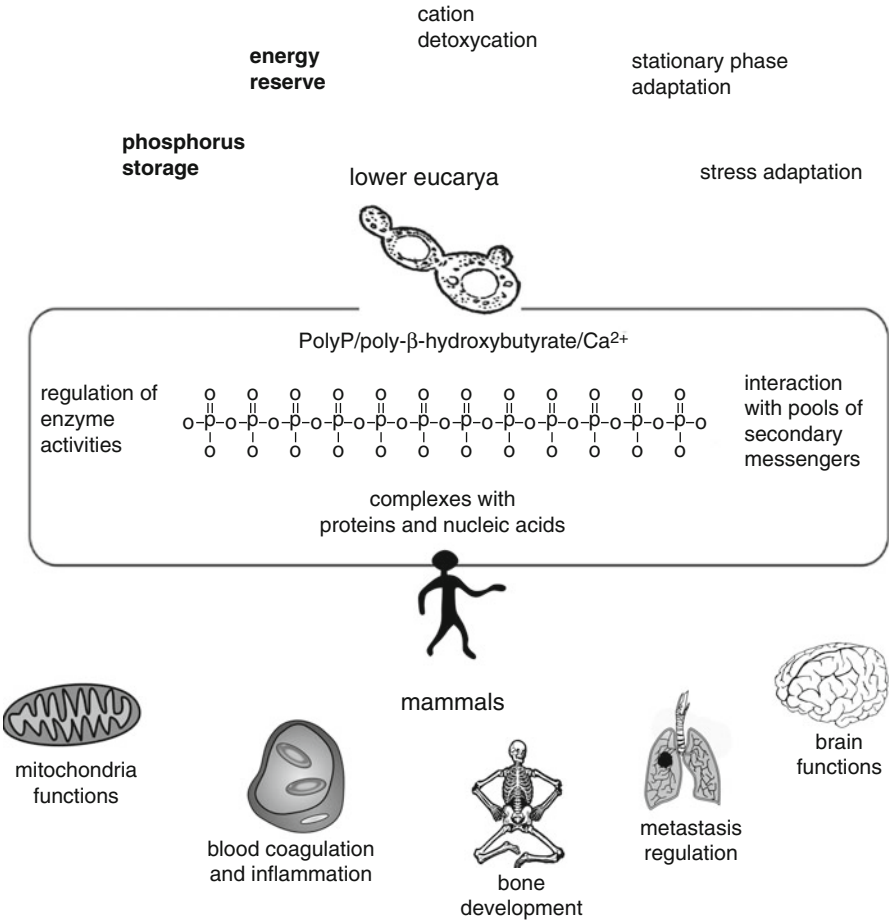

Fig. 1 PolyP functions in lower and higher eukaryotes

phosphorus mineralization in microorganisms and the bone apatite formation in mammals (Fig. 2). Individual stages of these processes are characterized by predominance of either the uptake of phosphorous mineral compounds from the medium or their release from the cells (and/or release from the cells in case of death). Phosphate concentration from the medium is accompanied by local accumulation of inorganic polyphosphates in the cells. Under varying environmental conditions or cell death, polyP is released into extracellular medium and hydrolyzed by phosphatases; apatite is formed from the released P_i in the presence of calcium ions.

The study of evolution of polyP-metabolizing enzymes is one of tasks necessary for understanding the normal and pathological processes in human organism associated with polyP metabolism. The eukaryotes have no polyphosphate kinases that are homologue to bacteria. No homologue of vtc4 polyphosphate synthase was found in

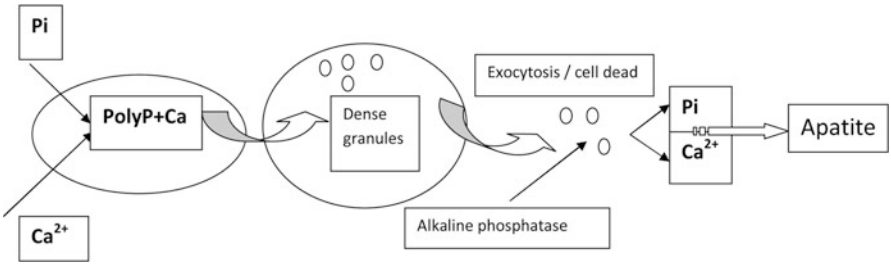

Fig. 2 The role of polyP in the apatite formation after death of the cells of polyP-accumulating bacteria and in the bone formation in mammals

mammals. So far the enzymes of polyP synthesis in higher eukaryotes have not been identified. The evolutionary approach and modern genomic methods may be a key to the solving of this problem.

References

Barge LM, Abedian Y, Doloboff IJ et al (2015) Chemical gardens as flow-through reactors simulating natural hydrothermal systems. J Vis Exp 105. doi:10.3791/53015

Beck A, Orgel LE (1965) The formation of condensed phosphate in aqueous solution. Proc Natl Acad Sci U S A 54:664–669

Holm NG, Baltscheffsky H (2011) Links between hydrothermal environments, pyrophosphate, na(+), and early evolution. Orig Life Evol Biosph 41(5):483–493

Miller SL, Parris M (1964) Synthesis of pyrophosphate under primitive earth conditions. Nature 204:1248–1249

Norris V, Reusch RN, Igarashi K, Root-Bernstein R (2014) Molecular complementarity between simple, universal molecules and ions limited phenotype space in the precursors of cells. Biol Direct 10(1):28

Osterberg R, Orgel LE (1972) Polyphosphate and trimetaphosphate formation under potentially prebiotic conditions. J Mol Evol 1:241–252

Pasek MA (2008) Rethinking early Earth phosphorus geochemistry. Proc Natl Acad Sci U S A 105(3):853–858