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Cell Stress by Phosphate of Two Protozoa *Tetrahymena thermophila* and *Tetrahymena pyriformis*

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Abstract

Phosphorus is one of the bioelements most needed as a compound cell by living organisms. Phosphorus is involved in several pathologies: in human with bone and kidney diseases, in mammals with metabolism disorder (glucose, insulin…), in microorganisms whose phosphorus is involved in cell growth. Phosphorus has various forms including pyrophosphate, a by-product of multiple pathways of biosynthesis. Enzymes that hydrolyze pyrophosphate are called inorganic pyrophosphatases (PPases). Two major types of inorganic pyrophosphatases are distinguished: the soluble pyrophosphatases (sPPases) and the membrane pyrophosphatases (mPPases or H^+/Na^+-PPases). They play a key role in the control of intracellular inorganic pyrophosphate level and produce an important ions gradient (H^+ or Na^+) to the cells. In this work, we primarily focused on the physiological study in a phosphate-poor medium of two models *Tetrahymena thermophila* and *Tetrahymena pyriformis*, following the mobility, the growth and the morphology of cells. Secondly, we evaluated the enzymatic activity of soluble and membrane pyrophosphatases in both species grown in the same complex medium. A decrease of cell growth is correlated with unusual morphologies and different mobility in the stress medium. The measurement of soluble and membrane inorganic pyrophosphatases activities also shows a decrease which illustrates the lack of phosphate found in the stress medium. Deficiency of phosphate is a limiting factor for protozoan growth. These results indicate that Tetrahymena can be used as a model of cellular stress and consists of a target to study inorganic pyrophosphatases for a better understanding of phosphate cycle in higher organisms.

Keywords

Phosphorus, Pyrophosphatases, NBRIP, Tetrahymena

1. Introduction

Phosphorus is an essential element of cellular metabolism. It is an important component of cells structure, incorporating in the skeleton of molecules to form phosphate groups such as adenosine triphosphate (ATP), nucleic acids (DNA, RNA), phospholipids and phosphoproteins of animals and plants cells. Phosphorus is also a component of bones and teeth, its absorption *in vivo* is ensured by vitamin D and its serum level is regulated by renal reabsorption [1].

In Humans, inorganic phosphate is involved in various pathological disorders such as bone, kidney, and vascular calcification [2]. Indeed, a deficiency of phosphate is found in rickets disease from children, osteomalacia in adults and Toni-Fanconi syndrome which leads to bone loss [3] [4]. In mammals, it is shown that a low phosphate diet alters glucose metabolism [5] [6] and the expression of insulin-sensitive genes [7] [8]. In addition, it is shown that phosphate increases oxidative stress such as Klotho deficiency in mice, causing phosphate retention and impairment of cognition due to increased oxidative damage and apoptosis in Hippocampus neurons [9]. In microorganisms, a deficiency of phosphate in the culture medium induces a cell cycle arrest in *Saccharomyces cerevisae* [10].

Phosphorus is present in several forms, in nature with its most oxidized form $\left(PO_4^{2-}\right)$ and generally bound to metal ions which are components of various rocks and minerals; in living cells whose phosphorus is transported as orthophosphate anion $\left(H_2PO_4^{2-}\right)$, where it is a part of phosphate groups such as pyrophosphate $\left(PO_3^{2-}\text{-O-}PO_3^{2-}\right)$. Pyrophosphate is a by-product of numerous synthetic reactions (nucleic acids, proteins, polysaccharides) and plays an important role of cellular metabolism. Consisting of two orthophosphate groups united by a phosphoanhydride bound, pyrophosphate constitutes a chemical grouping, whose fundamental binding energy is stored for energy requirements of the cell. However, intracellular inorganic pyrophosphate is constantly regulated and the hydrolysis of this product indicates that the biosynthetic reactions proceed in the right direction [11] [12]. It is also shown that an intracellular pyrophosphate deregulation is associated with various diseases [13].

Inorganic pyrophosphatases are enzymes that hydrolyze inorganic pyrophosphate into orthophosphate. They are distinguished in two groups: soluble pyrophosphatases (sPPases) and membrane pyrophosphatases (mPPases or H^+/Na^+-PPases). Both enzymes are heterologous which have difference in structure and amino acid sequences, but they have similar patterns in catalytic site sequences [14]. Soluble pyrophosphatases are ubiquitous enzymes that play a key role in phosphorus metabolism by catalyzing the hydrolysis of metabolically

produced pyrophosphate. According to their omnipresence, sPPases are important in case of absence an accumulation of pyrophosphate causes metabolic dysfunctions [15] [16]. sPPases consist of three families: family I most frequent and found in most eukaryotic, archaea and bacteria organisms; family II sPPases found in some bacteria, archaea and primitive eukaryotes; the unexplored family III found in some bacterial species [17] [18]. On the one hand, family I sPPases are distinguished from family II sPPases by their activation cofactor: Mg^{2+} is the main cofactor of sPPases whereas Mn^{2+} is the main cofactor of sPPases II [19] [20]. On the other hand, the sequences of both proteins are not similar, nevertheless the active site of the two enzymes remains conserved [19] [21]. In addition Family I sPPases are more sensitive to fluoride than family II sPPases [22] [23].

Soluble pyrophosphatases are essential in metabolic reactions of bacteria and yeasts [16] [24]. They are involved in bacteria growth such as *Escherichia coli*. A dysfunction of sPPases leads to the cell cycle arrest and death of fermenting yeast [25]. Similarly, soluble pyrophosphatases are overexpressed in breast cancer cells [26]. The structure and the function of these enzymes are important in biomedical field because family II pyrophosphatases are specific to certain pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus mutans* and *Bacillus anthracis*, which may be potential targets for discovery and development of new antibacterial products [27].

Membrane PPases are ionic pumps and found in plants, algae, some protozoa, bacteria, archaea. They produce a proton (H^+-PPases) or sodium (Na^+-PPases) gradient by coupling the hydrolysis energy of pyrophosphate [28]. H^+-PPases are found in prokaryotes (vesicles, acidocalcisomes) [29] [30], protists and eukaryotes (vacuolar plant membranes, acidocalcisomal membranes of protists) [31] [32]. H^+-PPases provide important energy reserves to organisms, especially under stress conditions and lack of energy [30] [33]. Na^+-PPases are mainly encountered in prokaryotes.

Membrane pyrophosphatases provide an ion gradient under stress conditions, when ATP is almost absent [30] [34]. These enzymes do not appear to be present in mammals but could be potential targets for vaccines and drugs development against parasitic protists.

However, inorganic pyrophosphatases are rarely studied in protozoa, particularly in ciliates, which are the first used organisms to understand the genetic phenomenon of higher organisms. Ciliates are also characterized by a different evolution from commonly studied organisms in the opistokont lineage [35]. The protozoan Tetrahymena is one of the most studied ciliated organisms and constitutes a eukaryotic cell model. *Tetrahymena thermophila* is a species widely used in cell physiology and molecular genetics studies which have led to the acquisition of much fundamental knowledge in biology [36] [37]. The species *Tetrahymena pyriformis* is also a very useful species in physiological and toxicological studies [38]. The protozoan Tetrahymena combines the biological com-

plexity of eukaryotes and the accessibility of unicellular organisms. It is introduced very early into the laboratory due to the easy culture in axenic media, the ideal length for studies under light or electronic microscope (30 - 50 μm).

In this work, we primarily focused on the physiological study in a phosphate-poor medium of two models *Tetrahymena thermophila* and *Tetrahymena pyriformis*, following the mobility, the growth and the morphology of cells. Secondly, we evaluated the enzymatic activity of soluble and membrane pyrophosphatases in both species grown in the same complex medium.

2. Materials and Methods

2.1. Strains and Culture Conditions

The strains *Tetrahymena thermophila* SB1969 and *Tetrahymena pyriformis* used in our study were cultured axenically in the standard medium (PPYE) containing proteose peptone (1.5%, w/v) and yeast extract (0.25%, w/v) [39]. The culture medium was inoculated with 1% (v/v) of *Tetrahymena thermophila* or *Tetrahymena pyriformis* preculture in the same medium. Tetrahymena thermophila cells were incubated at 32˚C without shaking, while *Tetrahymena pyriformis* cells were incubated at 28˚C without shaking. Medium without strains were also prepared in same condition to control eventual contamination.

2.2. NBRIP Medium

National Botanical Research Institute Phosphate medium (NBRIP) [40] was aseptically prepared and represent the stress-medium used in our study. NBRIP is deprived of phosphate and is composed of: $MgCl_2 \cdot 6H_2O$ (5 g/l); $MgSO_4 \cdot 7H_2O$ (0.25 g/l); KCl (0.2 g/l); $(NH4)_2SO_4$ (0.1 g/l); glucose (10 g/l); Ph = 7 ± 0.2. The culture medium is inoculated with 1% (v/v) of *Tetrahymena thermophila* or *Tetrahymena pyriformis* preculture from PPYE medium and incubated at 32˚C (*Tetrahymena thermophila)* or 28˚C (*Tetrahymena pyriformis*) without shaking. Medium without strains were also prepared in same condition to control eventual contamination.

2.3. Microscopic Observation

The behavior of both species is analyzed into PPYE and NBRIP culture media under light microscope. This study is carried out by sterilely sampling at 3H interval time. Samples are prepared and slide-based, and observed at objective ×10 with Leica DM 500 and A.KRÜSS Optronic microscopes. Protozoan growth is also verified in both culture media by absorbance measurement at 600 nm using Hitachi U-1100 spectrophotometer. The optical zero corresponds to the control media without strain.

2.4. Proteins Extraction by Glass Beads

Cells were harvested at 6000 g for 10 minutes (Beckman Coulter Avanti J-25)

and suspended in Buffer A containing 10 mM Tris-HCl pH8, 10% glycerol (p/v), 1 mM EDTA pH8, 1 mM PMSF, 10 mM β-mercaptoethanol (at ratio of 1 g cells/5 ml Buffer A), in which 1 g of glass beads (0.4 - 0.6 mm of diameter) was added. Cells were broken through the beads by vortexing in 8 cycles (1 minute agitating-1 minute in ice). Then, beads and unbroken cells were removed by centrifugation at 700 g for 10 minutes (Beckman Coulter Allegra™ 21 Centrifuge). Soluble and membrane fractions were obtained by centrifuging the total extract at 120.000 g for 40 minutes (Beckman KL-80 Utracentrifuge). The supernatant corresponds the soluble crude extract and the pellet was washed in two steps: with Buffer B (60 mM Tris-Hcl pH 8; 12% glycerol; 0.72 M KCl; 1 mM $CaCl_2 \cdot 2H_2O$; 1 mM PMSF) and centrifugation at 120.000 g/30 minutes–with Buffer A and centrifugation at 120.000 g/30 minutes. The final obtained pellet was suspended in Buffer A and corresponds to membrane crude extract.

2.5. Determination of Proteins Concentration

Proteins concentration was estimated according to Bradford technique [41]. Bovine Serum Albumin (0.1 mg/ml) was used as standard protein.

2.6. Pyrophosphatases Activity Assay

The determination of enzymatic activity is based on the method established by Rathbun and Betlach [42] which consist to measure released phosphate Pi after the hydrolysis of pyrophosphate PPi. The released phosphate form with molybdate ammonium a phosphomolybdate complex and the latter is reduced by stannous chloride giving a blue color. A unit of enzymatic activity corresponds to the amount of enzyme that hydrolyzes 1 µmol of PPi.

2.6.1. Soluble Pyrophosphatases Activity

Measurement of enzymatic activity was carried out in a reaction mixture (200 µl) containing 50 mM Tris-HCl pH 7.5; 2 mM $MgCl_2$; 0.5 mM Fluoride sodium (optional); adequate volume of soluble crude extract. The reaction was started with addition of 1mM Na4PPi and incubation at 30˚C for 10 minutes. Subsequently the reaction was stopped by adding the stop solution containing 37% Formaldehyde-3.5 M Acetate acid-30% Trichloroacetic acid). Then, ammonium molybdate (0.2% p/v) and stannous chloride (0.16% p/v) were added and absorbance was measured at 735 nm after 10 minutes of incubation.

2.6.2. Membrane Pyrophosphatases Activity

Membrane crude extract was used to realize the measurement of mPPases activity. Thus, reaction mixture (200 µl) was composed of: 50 mM Tris-HCl pH 7.5; 2 mM $MgCl_2$; 0.1 M KCl (optional); adequate volume of sample. The reaction was started with addition of 1 mM Na4PPi and incubation at 30˚C for 10 minutes. The reaction was stopped by adding 670 µl of stop solution, ammonium molybdate and stannous chloride were added and absorbance was measured 10 minutes later at 735 nm.

3. Results and Discussion

3.1. Analysis on Morphology, Mobility and Growth of Protozoa

The behavior of the two species *Tetrahymena thermophila* (TT) and *Tetrahymena pyriformis* (TP) is followed in PPYE and NBRIP culture media as described in material and methods. The cells are inoculated at 1% (v/v) and incubated at 32˚C (TT) or 28˚C (TP). Samples of each culture medium are taken sterile and three parameters are studied: density, mobility and morphology. The Table 1 summarizes the results obtained from various observations under light microscope.

There is a high cell density the normal PPYE medium while a low density of protozoa is observed in NBRIP medium. This difference is accompanied by a change in mobility: protozoa move quickly in the PPYE medium and slowly in the NBRIP medium. As regards the cellular structure, the cells observed in the PPYE medium present a pear-shaped characterizing the normal cells of Tetrahymena and move very quickly in anarchic direction (Figure 1). In NBRIP medium, except of some pear-shaped protozoa that move slowly (Figure 2(a)), several cells are found in atypical form and are immobile (Figure 2(b)). These parameters measured in both culture media do not show a significant difference between both species TT and TP.

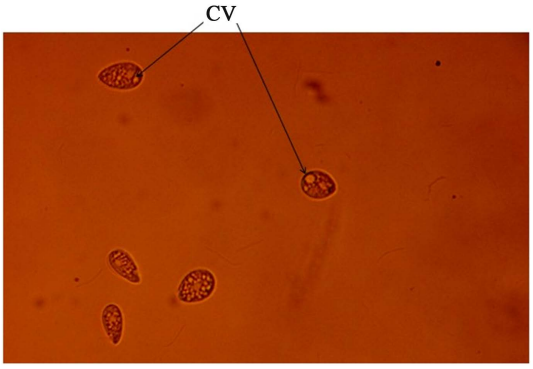

Figure 1. Microscopic image of *Tetrahymena thermophila* in PPYE medium (×10). CV = Contractile Vacuole.

Table 1. Parameters illustrating the behavior of both species *T. thermophila* and *T. pyriformis* according to culture media after 72 hours of incubation.

	Tetrahymena thermophila			*Tetrahymena pyriformis*		
	Density	Morphology	Mobility	Density	Morphology	Mobility
PPYE	+++	Pear-Shaped	Rapid moving	+++	Pear-Shaped	Rapid moving
NBRIP	+	Atypical Form >>> Pear-Shaped	Slow moving	+	Atypical Form >>> Pear-Shaped	Slow moving

(+: presence; >: higher than).

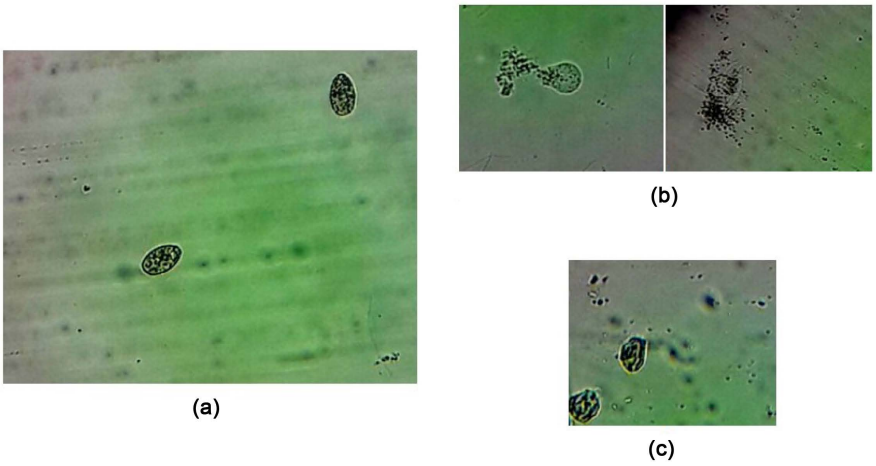

Figure 2. Microscopic images of *Tetrahymena thermophila* in NBRIP medium (×10). (a) Pear shaped; (b) Broken cell; (c) Degenerate cell.

Structurally, there is a difference in the architecture of cells vacuoles from PPYE and NBRIP media. The vacuoles are well defined and visible in the normal medium (**Figure 1**) while they are narrower in NBRIP medium (**Figure 2(a)**). Also the contractile vacuole is clearly visible in the PPYE cells and absent in the cells of the NBRIP medium. The contractile vacuole is an osmoregulatory organelle for Tetrahymena [43] and his absence may illustrate the disrupting cells observed in NBRIP medium (**Figure 2(c)**).

NBRIP medium is a medium known for its phosphate deficiency. It is generally supplemented with complex phosphate to evaluate the capacity of phosphate solubilizing microorganisms. We found no difference of growth in NBRIP medium or NBRIP supplemented with tricalcium phosphate (TCP), indicating that Tetrahymena has no solubilizing activity of complex phosphate. Thus, protozoa are confronted with a medium low in phosphate and limited in nutrients.

Although the growth in NBRIP medium is slowed down and illustrates the low density of the cells observed under an optical microscope, a resistance phenomenon is observed in this medium because viable cells are found even after 144H of culture. This resistance can be explained by the fact that protozoa have polyphosphate reserves [44] which can be used in the event of stress. In addition protozoa have pyrophosphate granules reserves whose appearance depend on calcium and magnesium rate and accumulate during the stationary phase or in case of large amount of phosphate in culture medium [45].

3.2. Enzymatic Activity Assays

Inorganic pyrophosphatases play an important role in the hydrolysis mechanism of inorganic pyrophosphate and their activity depends on cellular living conditions. Enzymatic activity of soluble pyrophosphatases (sPPases) and membrane pyrophosphatases (mPPases) is measured in both species *Tetrahymena thermophila* (TT) and *Tetrahymena pyriformis* (TP), according to standard (PPYE) or complex (NBRIP) media.

In standard medium, mPPases dependent on KCl activity is about 25 mU/mg in both species. In NBRIP medium, enzymatic activity also dependent on KCl decreased to 10 mU/mg (Figure 3).

The activity of sPPases under standard medium is in the order of 30 mU/mg in TT and 10 mU/mg in TP whereas in the NBRIP medium the activity of sPPases decreased considerably in TT (4 mU/mg) and TP where it is absent (Figure 3). These activities are sensitive to fluoride and seem to correspond to the family I sPPases activity.

Soluble pyrophosphatases play an important role in the regulation of pyrophosphate by preventing its accumulation which disturbs the metabolic reactions [15] [16]. The mPPases constitute ion pumps and use pyrophosphate as a substrate to create a gradient of Na^+ or H^+ ions [28] essential for cellular metabolism. Under stress, cellular metabolism is reduced and a decreased pyrophosphatases activity is normal because of the restriction of metabolic reactions. The absence of soluble pyrophosphatases activity in the NBRIP stress medium results in a small amount of pyrophosphate released through metabolic pathways. Membrane pyrophosphatases activity, however low, ensures the maintenance of ion gradient essential for cell survival. This decline accompanied a reduction of the growth rate of protozoa, the appearance of form of resistance and broken cells by osmotic pressure.

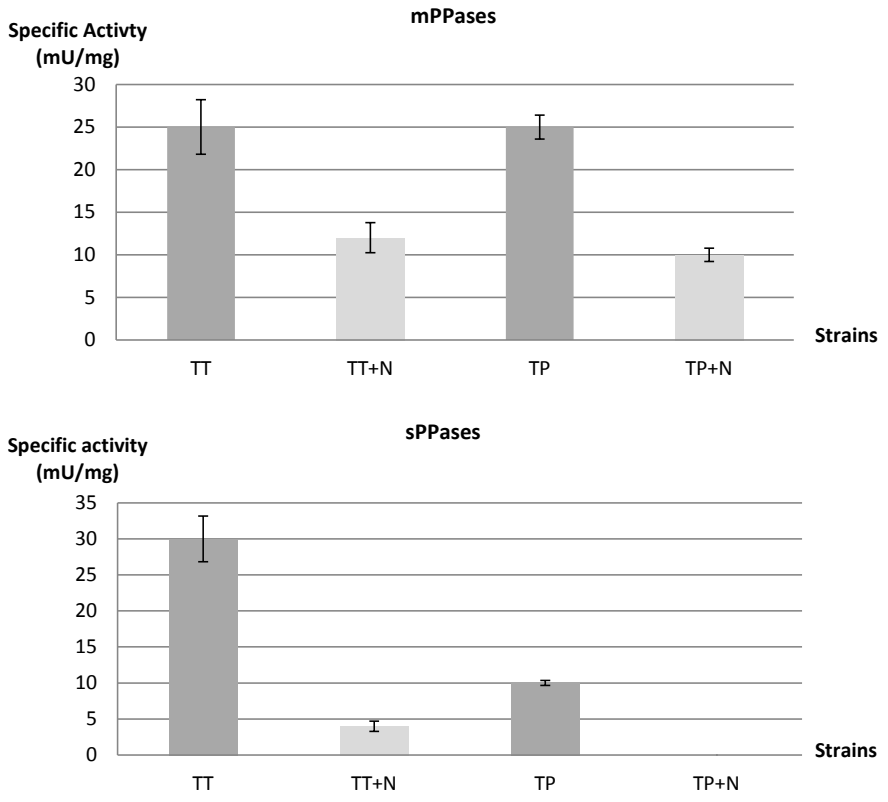

Figure 3. sPPases and mPPases activities of both species *Tetrahymena thermophila* (TT) and *Tetrahymena pyriformis* (TP) in PPYE (TT, TP) and NBRIP (TT + N, TP + N) media.

Phosphate, by its omnipresence in cells of living organisms, is therefore of capital interest particularly in cellular organelles such as mitochondria which are the site of several biosynthetic reactions involving the hydrolysis of pyrophosphate (activation of fatty acids, [46]). The pyrophosphate hydrolysis in these organelles is essentially assured by soluble inorganic pyrophosphatases (sPPases) and a decrease of metabolic reactions as NBRIP medium also shows a decreased activity of these enzymes.

Protozoa survival in the NBRIP medium can be explained by the fact that the cells have reserves of phosphate (polyphosphates, pyrophosphate granules) which are used to ensure their growth and compensate the lack of nutrients in stress medium. It is shown that the hydrolysis of these reserves is not assured by inorganic pyrophosphatases [45] and does not contradict the low pyrophosphatases activity found in NBRIP medium. Nevertheless, a subsequent study of these enzymes involved in the hydrolysis of these reserves would be of great interest for understanding the phosphate cycle from the Tetrahymena model.

4. Conclusion

This study shows the importance of phosphate as a cellular compound. Cellular damage that phosphate can generate is elucidated in the protozoa *Tetrahymena thermophila* and *Tetrahymena pyriformis* are considered as unicellular eukaryotic models. Our results indicate that the lack of phosphate affects the morphology, mobility, growth of protozoa and induces a decrease in inorganic pyrophosphatases activity. Proteomics and transcriptomics studies of these enzymes of interest in Tetrahymena will be important for further investigations for a better understanding of phosphate cycle in higher organisms.

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