New insights into the in situ microscopic visualization and quantification of inorganic polyphosphate stores by 4',6-diamidino-2-phenylindole (DAPI)-staining

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Introduction

Inorganic polyphosphate (PolyP) is a widely distributed biological polymer composed of phosphate residues linked by high-energy phosphate bonds, and found in every tested organism. Several biological functions have been described for PolyP, including a role as phosphate reservoir, metal homeostasis, regulation of transcription factors and transcription fidelity, as well as regulation of several enzymatic activities.2 More recently, studies on the role of PolyP in vertebrate models revealed the participation of PolyP stores in platelet activation and intrinsic coagulation,3,4 cancer metastasis,5,6 activation of the fibroblast growth factor (FGF) signaling and a role in stem cell differentiation.3,10

Although extensively demonstrated in several subcellular compartments such as nuclei, mitochondria, as well as in the cytoplasm of different cells, millimolar concentrations of PolyP are commonly found inside PolyP-rich organelles. A wide variety of PolyP-rich organelles has been described, but recent studies have pointed that at least some of these compartments probably derive from a common evolutionary step and are similar to the protozoan acidocalcisome,11 the best studied PolyP-rich organelle. It has been suggested that PolyP stored in acidocalcisomes have a role in protozoan virulence12,13 as well as in the maintenance of osmotic balance14 and metal homeostasis.15 Together with protozoan acidocalcisomes, bacterial volutin granules are also considered one of the most studied types of PolyP-rich structures. Although volutin granules have been known for a longer period, it was only after the first description of the acidocalcisome in trypanosomes that the concept that this organelle might have a membrane unit containing specific transporters was established in bacteria.16,17 After these initial studies, several other organisms were shown to contain PolyP-rich acidocalcisome-like organelles, including eggs of invertebrates such as insects,18,19 sea urchin,20 and vertebrates (chicken),21,22 models where the isolation of subcellular fractions of acidocalcisomes was readily demonstrated.

The significant biological relevance of PolyP in cell metabolism has lead to the establishment of different protocols for PolyP visualization and quantification, an important step towards a better comprehension of the roles played by this polymer in different biological models. In this regard, the use of 4',6-diamidino-2-phenylindole (DAPI), first developed as a nucleic acid sensor, both in spectrofluorimetry.23,24,25

Abstract

Inorganic polyphosphate (PolyP) is a biological polymer that plays important roles in the cell physiology of both prokaryotic and eukaryotic organisms. Among the available methods for PolyP localization and quantification, a 4',6-diamidino-2-phenylindole (DAPI)-based assay has been used for visualization of PolyP-rich organelles. Due to differences in DAPI permeability to different compartments and/or PolyP retention after fixation, a general protocol for DAPI-PolyP staining has not yet been established. Here, we tested different protocols for DAPI-PolyP detection in a range of samples with different levels of DAPI permeability, including subcellular fractions, free-living cells and cryosections of fixed tissues. Subcellular fractions of PolyP-rich organelles yielded DAPI-PolyP fluorescence, although those with a complex external layer usually required longer incubation times, previous aldehyde fixation and/or detergent permeabilization. DAPI-PolyP was also detected in cryosections of OCT-embedded tissues analyzed by multiphoton microscopy. In addition, a semi-quantitative fluorimetric analysis of DAPI-stained fractions showed PolyP mobilization in a similar fashion to what has been demonstrated with the use of enzyme-based quantitative protocols. Taken together, our results support the use of DAPI for both PolyP visualization and quantification, although specific steps are suggested as a general guideline for DAPI-PolyP staining in biological samples with different degrees of DAPI and PolyP permeability.
mented among several publications that suggested a number of possible routines for DAPI-PolyP labeling and visualization. Information available in most catalogs and product data sheet classify DAPI (different varieties) as compounds slightly permeable to cell membranes, although not systematically studied in different cell types. The degree of permeability presumably depends on the structural characteristics of the different cell surfaces in different organisms. In this regard, although a few studies have shown the spectroscopic properties of analytical grade sodium PolyP-DAPI fluorescence for quantification purposes, quantitative analysis of PolyP mobilization using DAPI as a probe in biological samples has not yet been reported. In the present study, we evaluated the effect of different routines of DAPI incubation in subcellular fractions, whole cells (containing or not cell walls), and OCT-embedded semi-thin sections in order to ascertain a unified guideline for PolyP labeling and detection. A semi-quantitative fluorimetric assay for the analysis of PolyP mobilization in PolyP-enriched extracts obtained from biological samples is also shown and provides a fast protocol for PolyP quantification.

Materials and Methods

Animals, cell culture and strains

Trypanosoma cruzi epimastigote forms (Y strain) were grown axenically in liver infusion tryptose (LIT) medium supplemented with 10% fetal calf serum as previously described. Cells were collected by centrifugation at 350 g after four days of cultivation. Trypanosoma brucei procyclic forms (strain 427) were maintained at 27-28°C in SDM-79 medium supplemented with 10% fetal bovine serum. Cells were harvested by centrifugation at 350 g after two days of cultivation. The attainment of Eimeria spp. oocysts from chickens was approved by the Ethics Committee of the Biology Institute of the State University of Campinas (UNICAMP) under the protocol number 1084-1, according to the Brazilian federal law (11.794/2008, Decrete n. 6.899/2009) that is based on the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences, USA, and the Australian Code of Practice for Care and Use of Animal for Scientific Purpose. All animals received humane care in compliance with the above-mentioned guidelines used by the Ethics Committee to approve the protocol. Briefly, four-week-old chickens were inoculated with 1x10⁵ sporulated oocysts of Eimeria acervulina (Cu strain) and 5x10⁵ Eimeria tenella (Pa strain) oocysts, as previously described. Oocysts were isolated from feces and sporulated after one week of incubation. Sporozoites were obtained from oocysts by mechanic rupture, enzymatic treatment, and purification by anion-exchange chromatography using DE-52 cellulose columns. Euglena gracilis wild strain was cultivated axenically at room temperature and constant illumination as previously described. Rhodnius prolixus Stahl, 1859 (Hemiptera, Reduviidae) were reared in a colony maintained at 28°C and 70-80% relative humidity. The insects were fed with rabbit blood in an artificial apparatus as described before. Total egg homogenates (TEH) were prepared by disrupting the laid eggs and a fraction enriched in acidovalciosomes was obtained as described by Ramos et al. The final pellet was used as acidovalciosome-enriched fraction (acidovalciosome fraction). Anticarsia gemmatalis specimens were obtained as described; briefly, a colony was kept at 27°C and 70% relative humidity. Adults were maintained in a plastic cage and paper sheets were added for eggs deposition. After 24 h, eggs were transferred to a plastic box and left for egg hatching and larvae development. Larvae were fed as previously described, until they reached the fifth instar around the 10th or 11th day after hatching as detected by visual inspection.

PolyP staining in whole cells

For PolyP detection, living cells were incubated with 50 µg/mL DAPI for 30 min, or different times where indicated, at room temperature. Alternatively, Eimeria sporocysts were first fixed with 4% formaldehyde for 5-20 min at room temperature and permeabilized with 0.3% Triton X-100 for 5 min prior to DAPI incubation. Mechanically disrupted sporocysts were also prepared as described. Following DAPI incubation, samples were mounted on glass slides and observed with a Zeiss LSM 510 Meta NLO multiphoton microscope using two photon fluorescence excitation of 780 nm. A wavelength scan was performed in order to detect the most intense fluorescence wavelengths.

Fluorimetric analysis of PolyP

Time-lapse analysis of PolyP fluorescence was evaluated by washing cell suspensions with PBS and incubating with 100 µg/mL DAPI. After addition of DAPI, samples were immediately transferred to a microplate reader and the fluorescence intensity was registered over the course of 60 min, using excitation/emission wavelength of 350/450 (nucleic acid signal) and 350/550 (PolyP signal). Water-soluble PolyP fractions (WSF) were prepared from Periplaneta americana and R. prolixus eggs as described and both WSF, sodium phosphate glass - PolyP₂⁺, (Sigma Aldrich, St. Louis, MO, USA) – and plasmidial DNA were incubated with 20 µg/mL DAPI for 30 min at room temperature. Samples were analyzed with a spectrofluorometer at the excitation wavelength of 354 nm. Relative quantification of PolyP in R. prolixus eggs was performed as described. For comparison, PolyP samples were extracted as described and incubated with an excess of sePPX for 15 minutes at 37°C. Resulting Pi levels were analyzed with malachite green.
Results

PolyP-DAPI staining of subcellular isolated compartments and single cells

Subcellular fractions of PolyP-rich organelles were obtained as previously described and verified by electron microscopy analysis of whole mounts adhered to formvar-coated grids, showing the typical electron-dense characteristic of acidocalcisomes (Figure 1A). Additional confirmation was obtained by electron-probe X-ray microanalysis where high amounts of phosphorus and oxygen, bound to a number of cations, were detected (Figure 1B). PolyP stores in these fractions were rapidly stained (2-3 min at room temperature) using a range of 2-10 µg/mL DAPI and observed using a filter set optimized for DAPI-PolyP visualization (Figure 1C). Similar results were obtained using acidocalcisome fractions isolated from other organisms, such as PolyP-rich granules of chicken eggs (data not shown) and are consistent with those obtained from iodixanol differential sedimentation from other models.

DAPI-PolyP was also detected in whole cells of Trypanosoma cruzi (Figure 2A-B), a protozoan where both the size and number of T. cruzi PolyP-rich organelles (acidocalcisomes) are well known. Preparations of whole cell mounts seen in TEM showed electron dense organelles in the intracellular milieu (Figure 2A), presenting morphological similarities with the acidocalcisome-rich fractions depicted in Fig. 1A. X-ray microanalysis spectra confirmed the high content of phosphorus, oxygen and several cations (Figure 2A, inset), as previously described. When DAPI-PolyP staining was carried out in whole cells, higher concentrations of DAPI and longer incubation times were usually required. Incubation of intact cells with DAPI at the same conditions used for staining subcellular fractions (2-10 µg/mL or 5 min) did not result in satisfactory staining (data not shown). In contrast, 50 µg/mL DAPI for 10-20 min showed an intense staining pattern compatible with the presence and distribution of acidocalcisomes (Figure 2B) in these cells. This result suggested that the presence of external membranes or additional layers with different degrees of DAPI permeability may potentially diminish the penetration of the dye, therefore requiring slight modifications in the staining protocol. To verify whether these additional layers would actually interfere in PolYP-DAPI staining, we performed a PolYP detection assay under similar conditions in a range of unicellular organisms, i) containing plasma membrane with different properties, or ii) presenting additional cell or cyst walls. We first analyzed detection of PolyP stores in Euglena gracilis, known to present unique plasma membrane morphology supported by a dense fibrillar layer, the pellicular cortex. In Euglena cells, intracellular PolyP stores (acidocalcisome-like) were usually detected using higher concentrations of DAPI (50-200 µg/mL), following a 30 min incubation period at room temperature, without any further modifications in the protocol (Figure 3A).

To verify whether the presence of a cell wall would diminish DAPI penetration into the cells, we incubated Candida albicans cells with different concentrations of DAPI and followed the staining pattern over time. In general, although the additional cell wall layer seemed to reduce DAPI penetration in some cells, PolyP stores were readily visible using 50 µg/mL DAPI (Figure 3B). Eimeria parasites have also been shown to contain PolyP-rich organelles. These cells provided an interesting model for testing PolyP detection with DAPI, since the sporozoites (infective cells that contain only a limiting plasma membrane as a surface barrier) are found inside sporocysts that contain a cyst wall (each sporocyst contain two zoites). In contrast to yeast cells, PolyP detection in Eimeria sporozoites protected by a cyst wall inside sporocysts was not possible using the above mentioned protocols. In an attempt to enhance the access of DAPI to the intracellular milieu, sporozoites were submitted to i) gentle mechanical disruption/permeabilization as previously reported (Figure 3 C,D) or ii) Triton X-100 permeabilization followed by aldehyde fixation (Figure 3 E,F), before incubation with DAPI. Results showed an intense staining of PolyP-rich organelles (acidocalcisomes) inside the sporozoites. Although a previous step of aldehyde fixation has not significantly altered PolyP staining properties of DAPI in Eimeria cells, the same procedure seemed to impair DAPI-PolyP staining in other organisms, either by excluding DAPI penetration across the fixed cell wall or causing leaking of PolyP from the target organelles after chemical fixation (Figure 3B, inset). Nevertheless, it is important to note that in other yeast cell types such as the pathogenic fungi Cryptococcus neoformans, suc-
cessful PolyP-DAPI staining has been carried out without the previous step of aldehyde fixation (data not shown), suggesting that the structural organization of the cell wall may be important for the permeation of the dye. Additional time-lapse analyses showed that the staining intensity was time-dependent and varied with the cell type and the number and type of surface barriers. Fluorescence microscopy analysis of C. albicans incubated for different times showed acidocalcisomes became visible after 30 min and fluorescence intensity reached a plateau within 50 min (Figure 4A). Time-scale spectrofluorimetric analysis showed that in contrast to C. albicans, this plateau was reached in T. cruzi and T. brucei within 15-20 min, both for nuclei and PolyP staining (Figure 4 B,C).

**DAPI staining in tissues**

Several studies have shown that the inorganic content of PolyP stores is usually washed during different steps used in sample preparation for electron microscopy, resulting in empty organelles observed in transmission electron microscopy images from several models.24,47,48 This suggested that the preparation of semi-thin sections of PolyP-containing tissues for light microscopy would potentially require protocol adaptations to preserve the PolyP content. In this regard, preparation of semi-thin cryosections of OCT-embedded tissues allowed on one hand minimal sample handling and, on the other, the obtaining of sections containing the whole diameter of the PolyP-rich organelles, avoiding the leaking of PolyP from sectioned organelles, as observed in TEM preparations. Analysis of semi-thin sections of the midgut of the insect A. gemmatalis showed the presence of PolyP stores in some apocrine vesicles at the apical region of columnar cells (Figure 5A) as well as in vesicles occurring mainly around the goblet cell cavities (Figure 5B), as previously described.36,49

Results provided by different groups have shown that in situ DAPI-PolyP staining of PolyP granules may result in a slight variation of fluorescence emission peaks or colors (from green to yellow) (Figure 3).26,27 To check whether different organelles in the same organism might yield different emission peaks, a localized in situ wavelength scan of different PolyP-rich apocrine secretions in the midgut of A. gemmatalis was carried out using multiphoton microscopy. Results showed that different organelles generated similar fluorescence emission profiles, ranging from 516 to 592 nm, with a maximum around 528-549 nm (Figure 6, lines 1-3), which in general corresponded to DAPI-bound commercial sodium PolyP75 emission wavelengths (Figure 7A). Wavelength scans of con-

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**Figure 3.** Neither prior fixation nor permeabilization was needed for detecting PolyP by DAPI staining (50 µg/mL for 30 min) in intracellular stores in living cells from (A) E. gracilis presenting c: chlorophyll autofluorescence, or (B) C. albicans. Formaldehyde fixation prevented DAPI staining of the PolyP granules in C. albicans (B, inset). On the other hand, formaldehyde fixation followed by either mechanical lysis (C,D) or Triton X-100 permeabilization (E, F) was needed to visualize subcellular PolyP stores in Eimeria cells by DAPI staining. Scale bars: A, 10 µm; B, 10 µm; C, 5 µm; E, 5 µm.
trol areas did not generate similar fluorescence spectra (Figure 6, line 4).

Quantitative fluorimetric analysis of DAPI-PolyP staining in dynamic events

Spectrofluorimetric analysis of analytical grade sodium polyphosphate (PolyP) (Figure 7A) and PolyP extracted from *P. americana* WSF (Figure 7B) confirmed fluorescence spectra qualitatively different from DNA-rich (plasmid) samples (Figure 7B), supporting the use of fluorimetric methods for the quantitative analysis of DAPI-PolyP staining. Although some reports have suggested similar approaches with minor variations, a semi-quantitative measurements of PolyP mobilization in biological samples using DAPI as a probe were yet to be compared against results from more widely accepted methodologies such as scPPX quantification following PolyP binding onto glassmilk particles. Here, we measured PolyP mobilization by following DAPI-PolyP fluorescence levels in samples containing PolyP extracted from eggs of the insect *R. prolixus* during the early stages of development. Results showed that a strong mobilization of PolyP takes place during this period (Figure 7C) and were validated after a comparison against the well-established protocol for PolyP quantification based on the PolyP binding to silica powder in suspension followed by enzymatic PolyP hydrolysis into Pi by a recombinant yeast exopolypophatase, which showed similar profiles of PolyP mobilization during the early days of development.

Discussion

PolyP-rich organelles comprise the major PolyP reservoir in several eukaryotic models such as yeast and protozoans. PolyP stores were first detected with metachromatic reactions using basic dyes such as toluidine blue, methylene blue or neutral red, and were, thus, named metachromatic granules. These reactions generally depend on a previous step of specimen fixation, thus limiting experiments in live cells. Polymeric compounds other than PolyPs, such as nucleic acids, have also been shown to be stained with basic dyes, dragging the attention to the poor specificity of some dyes and the necessity for the use of markers with higher specificity. Tetracycline has been alternatively used to indirectly stain bacterial PolyP. Nevertheless, fluorescence mechanism involves binding of tetracycline to divalent cations (usually chelated by PolyP) within PolyP-rich organelles, providing a sensitive and selective method for PolyP detection.
viding signals of low specificity. Alternative methods such as the use of PolyP-binding domain of the *E. coli* exopolyphosphatase (PPBD) fused with an Xpress tag for the indirect detection of PolyP with the use of secondary antibodies have been reported. As yeast exopolyphosphatase (scPPX) is highly specific to PolyP, the method provides both specificity and sensitivity, although as PPBD is not commercially available, in lab production of PPBD requires protein engineering, bacterial strain transformation and protein expression and purification. In the last years, the use of DAPI as a PolyP-rich organelle sensor has gained popularity. It yields fluorescence at 525-550 nm after PolyP binding, displays a greenish-yellowish color and is both commercially available and present a fairly good sensitivity and specificity for the detection of PolyP-rich stores. Several microscope settings have been previously used for *in situ* detection of PolyP stores by DAPI likely resulting in a confusing scenario for the outcome researcher. As PolyP shifts DAPI emission from 450 nm to 525-550 nm, the fluorescence hue changes to a yellowish-green color. Thus, fluorescence emitted from nuclei can theoretically be discerned from fluorescence from PolyP using regular DAPI filters (data not shown), if color sensitive cameras are used. Nevertheless, emissions from nuclei often become too intense to enable differentiation from the weaker PolyP emissions. The DAPI filter system has thus to be modified and set to block wavelengths shorter than 500-515 nm. This will not prevent nuclei greenish emission area from being detected, yet it should block the most intense blue wavelengths and even allow detection by a monochromatic detection system. During our analyses, these settings more commonly provided optimal results suggesting that it should be used as a default condition for DAPI-PolyP.
Quantification of PolyP levels have been done either with the use of its metachromatic properties\(^{20}\) or using enzymatic assays mainly based on the hydrolysis of PolyP by scPPX\(^ {14,70}\) or incorporation of Pi from PolyP into ADP and synthesis of ATP by a polyphosphate kinase (PPK).\(^ {19}\) Again, the same issues described for detection of PolyP stores are present and the methods either lack specificity and sensitivity, or require recombinant enzymes that are not commercially available. Nevertheless, biotechnological applications of PolyP such as the removal of phosphorous and heavy metals from wastewater demands a faster and cheaper method of quantification that still maintain its reliability.\(^ {19}\) In this regard, some authors have approached on a method for DAPI-PolyP quantification.\(^ {20,29,30,57,71}\) Here we performed an evaluation of relative PolyP levels to ascertain PolyP mobilization on a time scale using fluorescence levels generated by DAPI as a probe. Again, fluorescence levels were measured using the maximum DAPI excitation wavelength (~350 nm). It has been suggested that DAPI-DNA and unbound DAPI signal are significantly removed by using 415 nm excitation, which is imperative to discern PolyP specific signals in conditions of low PolyP content. Our present results were compared with the scPPX-based routine, which remains the most widely accepted PolyP quantification method, and demonstrated no major discrepancy between both methods. This suggests that when significant levels of PolyP are present, therefore giving a proper specific PolyP signal when compared to unbound or DNA/RNA-bound DAPI signals, quantification using excitation wavelengths around 350 nm are allowed. Nevertheless, it has been demonstrated that smaller polymers (<5 Pi residues) do not yield DAPI fluorescence.\(^ {29}\) Interference by DAPI binding to contaminants may also account for imprecise quantification. In this regard, DAPI binding to RNA has been described,\(^ {44}\) and also shown that treatment of PolyP extracts with RNase into has a strong influence on DAPI-PolyP signals under specific circumstances.\(^ {72}\) Analysis of the level of interference of DAPI-RNA on the samples should help to establish how absolute quantification of PolyP by DAPI can be accomplished.

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