Rapid changes in polyphosphate content within acidocalcisomes in response to cell growth, differentiation and environmental stress in Trypanosoma cruzi*

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Abbreviations: BCECF, 2’,7’-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; fura 2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2’-amino-5’-methylphenoxy)-ethane-N, N’, N’-tetraacetic acid; polyP, polyphosphate; Pi,
orthophosphate; PPi, pyrophosphate, DAPI, 4’, 6-diamidino-2-phenylindole.

Inorganic polyphosphate (polyP) has been identified and measured in different stages of *Trypanosoma cruzi*. Millimolar levels (in terms of Pi residues) in chains of less than 50 residues long, and micromolar levels in chains of about 700-800 residues long, were found in different stages of *T. cruzi*. Analysis of purified *T. cruzi* acidocalcisomes indicated that polyPs were preferentially located in these organelles. This was confirmed by visualization of polyPs in the acidocalcisomes using 4’, 6-diamidino-2-phenylindole (DAPI). A rapid increase (within 2-4 h) in the levels of short and long-chain polyPs was detected during trypomastigote to amastigote differentiation and during the lag phase of growth of epimastigotes (within 12-24 h). Levels rapidly decreased after the epimastigotes resumed growth. Short- and long-chain polyP levels rapidly decreased upon exposure of epimastigotes to hypo-osmotic or alkaline stresses while levels increased after hyperosmotic stress. Ca2+ release from acidocalcisomes by a combination of ionophores (ionomycin, nigericin) was associated with the hydrolysis of short- and long-chain polyPs. In agreement with these results, acidocalcisomes were shown to contain polyphosphate kinase and exopolyphosphatase activities. Together, these results suggest a critical role for these organelles in the adaptation of the parasite to environmental changes.
There is considerable interest in developing novel chemotherapeutic approaches against *Trypanosoma cruzi*, the etiologic agent of Chagas’ disease that remains an important health problem in Mexico, Central and South America (1). Some of these approaches are oriented towards the identification of biochemical pathways that allow survival of the parasite and are absent in the host.

An unusual characteristic of *T. cruzi* in comparison with mammalian cells, is the storage of calcium in acidic organelles which were termed acidocalcisomes (2). Initially identified in intact or permeabilized cells (3), the organelles have been isolated (4, 5) and found to have a high density, a high content of phosphorus, calcium, magnesium, sodium, and zinc (4, 6) and a number of pumps and exchangers in their limiting membrane, among them a Ca$^{2+}$-ATPase, a vacuolar H$^+$-ATPase, and a vacuolar H$^+$-pyrophosphatase (V-H$^+$-PPase) (3-7). Recent studies have shown that the phosphorus in the acidocalcisomes is in the form of pyrophosphate (PPi) (8) and short-chain polyphosphate (polyP) (9). Acidocalcisomes are therefore similar to the volutin granules described in other microorganisms (10-14). Although volutin granules were first described almost one hundred years ago (13), they have not been investigated concerning the presence of proton or calcium pumps in their limiting membrane. This is despite the fact that they were known to be acidic and to contain large amounts of calcium (12). The presence of these organelles in many microorganisms such as bacteria, fungi, algae, and protozoa and their apparent absence in mammalian cells makes them promising targets for chemotherapy.

Our previous $^{31}$P-NMR findings of large amounts of inorganic pyrophosphate and
short-chain polyP in T. cruzi (8, 9) are extended here to report the biochemical identification of the polyPs present in extracts of different stages of T. cruzi and isolated acidocalcisomes together with the first report of the presence of long-chain polyP and enzymatic activities involved in its synthesis and degradation, in these organisms. Our results indicate that the concentration of polyPs changes drastically during growth and differentiation of these parasites and that polyPs are rapidly mobilized under osmotic or alkaline stresses. Also, Ca\(^{2+}\) release from acidocalcisomes is associated with hydrolysis of polyP. The enzymatic activities required for these rapid changes, polyP kinase and exopolyphosphatase are present in the acidocalcisomes. The rapid mobilization of Ca\(^{2+}\) and polyP from acidocalcisomes suggests a critical role for these organelles in the adaptation of the parasite to environmental changes.

**EXPERIMENTAL PROCEDURES**

*Culture methods*— T. cruzi amastigotes and trypomastigotes (Y strain) were obtained from the culture medium of L6E9 myoblasts as we have described before (15). T. cruzi epimastigotes (Y strain) were grown at 28 °C in liver infusion tryptose (LIT) medium (16) supplemented with 10% newborn calf serum. Protein concentration was determined using the Bio-Rad protein assay. Trypomastigotes were induced to transform into amastigotes axenically as described previously (15).

*Materials*— Leupeptin, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
(E64), Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), ATP, ionophores except ionomycin, and reagents for marker enzyme assays, polyphosphates and phosphate glass (also known as sodium insoluble metaphosphate) were purchased from Sigma Chemical Co (St. Louis, MO). Silicon carbide (400 mesh) was bought from Aldrich (Milwaukee, WI). 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and ionomycin (free acid) were from Calbiochem (San Diego, CA). Pepstatin came from Boehringer Mannheim (Indianapolis, IN). Iodixanol (40% solution [OptiPrep], Nycomed) and Dulbecco’s PBS were obtained from Gibco BRL, Life Technology Inc. (Gaithersburg, MD). E. coli strain CA38 pTrcPPX1 was kindly provided by Prof. Arthur Kornberg, Stanford University School of Medicine (Stanford, CA).

Coomassie Blue protein assay reagent was from Bio-Rad (Hercules, CA). The HiTrap™ desalting columns were from Amersham Pharmacia Biotech AB (Upsala, Sweden). The HisBind column was from Novagen Inc. (Madison, WI). The EnzChek phosphate assay kit, and the tetraacetoxyethyl esters of fura 2 (1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2’-amino-5’-methylphenoxy)-ethane-N,N,N’,N’-tetraacetic acid), and BCECF (2’,7’-bis-(carboxyethyl)-5(and-6)-carboxyfluorescein), fura 2-AM and BCECF-AM, respectively, were from Molecular Probes, Inc (Eugene, OR). [32P]orthophosphate (8500 Ci/mmol) was obtained from New England Nuclear Life Sciences Product Inc. (Boston, MA). All other reagents were analytical grade.

*Isolation of acidocalcisomes*- Isolation of acidocalcisomes was done exactly as described before (5). Collected gradient fractions were assayed for hexokinase (glycosomal marker), acid phosphatase (lysosomal marker), alanine and aspartate aminotransferases (mitochondrial and cytosolic markers) and vacuolar pyrophosphatase (acidocalcisomal
marker) as previously described (4, 5).

Extraction of long- and short-chain polyP-

Cells (1 X 10^7- 1 x 10^8) were washed once with Dulbecco’s PBS and treated with methods to extract either long-chain, or short-chain polyP. Different samples were used for each method. Long-chain polyP extraction was performed as described by Ault-Riché et al. (17). For short-chain polyP extraction, the cell pellet was resuspended in ice-cold 0.5 M HClO₄ (2 ml/g wet weight of cells). After 30 min incubation on ice, the extracts were centrifuged at 3,000 x g for 5 min. The supernatants were neutralized by the addition of 0.72 M KOH/0.6 M KHCO₃. Precipitated KClO₄ was removed by centrifugation at 3,000 x g for 5 min and the extracted supernatant was used for polyP determination.

Purification of recombinant exopolyphophatase (rPPX1) from Saccharomyces cerevisiae-

E. coli strain CA38 pTrcPPX1 is an insertionally inactivated mutant for endogenous polyphosphate kinase and exopolyphophatase (18), containing a plasmid with the His-tagged rPPX1 gene from S. cerevisiae (19). This strain was grown to an A₆₀₀ of 0.6 in LB medium (1% tryptose, 0.5% yeast extract, 1% NaCl, pH 7.5). Addition of isopropyl β-D-thiogalactopyranoside (IPTG) (0.5 mM final concentration) induced the production of rPPX1. After incubation for 6 h, cells were harvested by centrifugation (4,000 x g for 15 min). The cells were resuspended in buffer I (50 mM Tris-HCl, pH 7.5, 0.1 M NaCl and 20 µM phenylmethylsulfonyl fluoride (PMSF) with 2 mg lysozyme/ml) and incubated on ice for 15 min. Then, the cells were sonicated (3 times for 20 sec at 20% intensity in a Branson sonifier, model 102c). The suspension was centrifuged for 30 min at 45,000 x g. The
supernatant was applied to a HisBind™ column that was equilibrated with buffer I and the column was washed with 3 column volumes of buffer I. Proteins were eluted with sequential additions of buffer I containing 50, 100, 200, 400, 800 mM imidazole and equilibrated with buffer I. Exopolyphosphatase activity was determined as in the analysis of polyP (below), using 0.6 mg/ml of polyP 15. PPX1 was eluted with buffer containing 50-100 mM imidazole, and the purity of the enzyme, estimated by SDS-PAGE, was over 95%.

Analysis of polyP- PolyP levels were determined from the amount of Pi released upon treatment with an excess of rPPX1. Aliquots of long- or short-chain polyP extracts (always less than 1.5 nmol) were incubated for 15 min at 37 °C with 60 mM Tris-HCl, pH 7.5, 6.0 mM MgCl₂ and 3,000 to 5,000 Units purified rPPX1 in a final volume of 75 µl. One unit corresponds to the release of 1 pmol of Pi per min at 37 °C. rPPX1 has been shown to be a powerful catalyst increasing the hydrolytic activity of a phosphoanhydride bond by 10¹¹-fold and is active on both polyP3 and longer chain polyP, as detected by treatment of radiolabeled polyP (20). Release of Pi was monitored by the method of Lanzetta et al. (21). The intracellular concentrations of polyP in different stages of *T. cruzi* were calculated from the respective cell volumes reported before (22). These values correspond to 30 µl/10⁹ epimastigotes and 12 µl/10⁹ amastigotes or trypomastigotes.

Electrophoretic analysis of polyP- Urea-polyacrylamide gels were prepared and stained with toluidine blue as previously described (23). Marker polyPs were obtained by electrophoresis of phosphate glass in 1.5% agarose gels. Four mm-long gel slices were eluted by centrifugation through Millipore Ultrafree-MC columns to obtain polyPs of
different sizes. The markers were localized by toluidine blue staining and their size was calculated by calibration with commercial polyP (Sigma).

**Isolation of [32P]polyP from epimastigotes** - Epimastigotes (2 x 10^7 cells) obtained at the exponential phase of growth were harvested by centrifugation, washed with 116 mM NaCl, 5.4 mM KCl, 0.8 mM Mg SO_4_, 5.5 mM glucose, 50 mM Tris-HCl pH 7.4, and resuspended in the same buffer supplemented with [32P]Pi (0.02 mCi/ml), and 50 mM NaH_2PO_4_. After incubation for 60 min at 28°C, the parasites were washed twice in the same buffer without [32P]Pi. Total poly P (short- and long-chain) extraction was performed as described by Kumble and Kornberg (23).

**Fluorescence microscopy** - Cells (5 x 10^7) obtained as described above (5) were washed twice with Dulbecco’s PBS. The pellet was resuspended in 2 ml of the same buffer and 45 µl of this suspension was incubated at room temperature with 10 µg 4’,6-diamidino-2-phenylindole (DAPI)/ml. After 10 min, the samples were mounted on a slide and observed with an Olympus model BX-60 epifluorescence microscope. Acidocalcisomal fractions (1 mg protein/ml) were incubated with DAPI as above. Olympus WIG (500-520 nm excitation; >580 nm emission) and Olympus WU (380-385 nm excitation; >420 emission) filters were used for polyP and DNA detection, respectively. The images were recorded with a CCD camera (model CH250; Photometrics Ltd., Tucson, AZ) and IPLab software (Signal Analytics, Vienna, VA) as described previously (24).

**Cell treatments** - Epimastigotes (1 x 10^9) were washed once in Dulbecco’s PBS,
resuspended in prewarmed isotonic, hypotonic or hypertonic media, and incubated at 30
°C. At the indicated times, aliquots were withdrawn, quickly transferred to an isotonic buffer
pre-equilibrated at 4 °C to stop the reaction, centrifuged, and the polyP content of the
pellets was quantified as described above. Isotonic medium (137 mM NaCl, 4 mM KCl, 1.5
mM K\(_2\)HPO\(_4\) and 8.5 mM Na\(_2\)HPO\(_4\)) was 300 ± 5 mosM, hypotonic medium (half of these
salts concentrations) was 150 ± 4 mosM and hypertonic medium (3/2 of these salts
concentrations) was 450 ± 6 mosM. All media contained in addition 20 mM Hepes, pH 7.4,
11 mM glucose, 1 mM CaCl\(_2\) and 0.8 mM MgSO\(_4\). Osmolarity of all solutions were checked
with an Osmette S automatic osmometer (Precision Systems Inc; Sudbury, MA). For the
alkaline or ionophore treatments epimastigotes or amastigotes (2.5 x 10\(^8\)) were washed
once with Dulbecco’s PBS and resuspended in 0.55 ml of 116 mM NaCl, 5.4 mM KCl, 0.8
mM Mg SO\(_4\), 5.5 mM glucose, 1 mM EGTA, and 50 mM Hepes pH 7.4 (buffer A). At the
times indicated, 40 mM NH\(_4\)Cl, 1 µM ionomycin or 1 µM nigericin were added. Aliquots of
50 µl were withdrawn at the times indicated and mixed with 500 µl of guanidine
isothiocyanate (GITC) lysis buffer (17) for long-chain polyP determination or with 300 µl of
ice-cold 0.5 M HClO\(_4\) for short-chain polyP determination as described above.
Spectrofluorometric determinations of amastigotes loaded with fura 2/AM or BCECF/AM
were performed as described previously (3). Data depicted in graphs are of single
representative experiments with data points given as means ± SEM.

*Acidocalcisomal synthesis and degradation of polyP*- To investigate polyP synthesis
by isolated acidocalcisomes, the isolated fraction (100 µg protein) (5) was incubated for 5 min at 37 °C in buffer A containing 0.1 mM PPi. ATP (1 mM) was then added and the preparation incubated for different times at 37 °C, after which 500 µl of GITC lysis buffer (for long-chain polyP determination) or 300 µl of ice-cold 0.5 M HClO₄ (for short-chain polyP determination) were added and polyP extracted and quantified as described above. To investigate polyP hydrolysis acidocalcisomes were suspended in a buffer containing 250 mM sucrose, 2 mM MgCl₂ and 50 mM Tris-HCl at the pHs indicated under Results. The suspension was divided in two samples and 1 mg/ml digitonin was added to one of them, while the other was kept as control. The suspension was then vortexed for about 1 minute and kept on ice for 5 min to measure tetrapolyphosphatase activity in the presence of 0.1 mM tetrapolyphosphate using a phosphate release assay kit (5). After the digitonin treatment aliquots were centrifuged at 10,000 x g for 5 min, to isolate the soluble content. The supernatant was collected and submitted to a second centrifugation step under the same conditions. Final supernatants and pellets were used for measurement of tetrapolyphosphatase activity at different pH and CaCl₂ concentrations.

RESULTS

PolyP abundance in different stages of T. cruzi- Long- and short-chain polyP were present in the different stages of T. cruzi; values for short-chain polyP were in the mM range and considerably higher in epimastigotes, which have also mM amounts of long-
chain polyP. Levels of 3.1 ± 1.4, 25.5 ± 5.1, and 54.3 ± 0.3 mM (in terms of Pi residues and calculated taking into account the cell volumes indicated under Experimental Procedures) in chains of less than 50 residues long, and levels of 82.5 ± 5.75, 130 ± 15, and 2889 ± 294.5 µM in chains of about 700-800 residues long, were found in trypomastigotes, amastigotes and epimastigotes, respectively.

PolyPs extracted from different stages of *T. cruzi* were electrophoresed by 6% urea-PAGE to determine their size distribution (Fig. 1A). Only one size class of polyP was detected in the three developmental stages: long-chain polyP of about 700-800 residues. The lack of detection of other polyPs suggests that the short-chain polyPs present in the different stages are too small to be recognized by toluidine blue (probably less than 5 residues) (25). In order to investigate the presence of short-chain polyPs, we labeled epimastigotes with [32P]Pi and the polyPs were extracted and electrophoresed using 6% urea-PAGE. The results are shown in Fig. 1B. Under these conditions labeled compounds that co-eluted with unlabeled commercial standards of polyP of about 5-15 Pi residues were obtained. This is consistent with our previous 31P NMR work (8, 9), in which high amounts of PPi, tri-, tetra-, and penta-phosphate were detected in epimastigotes and amastigotes of *T. cruzi*. Addition of a larger amount of material also permitted visualization of long-chain polyP (around 700-800 residues) (Fig. 1B, lane 2).

**Accumulation of polyP in acidocalcisomes**- The subcellular localization of the large amounts of polyP present in the parasites was investigated using two different methods. First, by subcellular fractionation, and second, by cytochemical techniques. Subcellular fractionation of epimastigotes of *T. cruzi* revealed that more than 95% of the short- and
long-chain polyPs were present in membrane fractions (10,000 x g pellet, Table 1). To investigate whether polyPs were present in acidocalcisomes, we isolated these organelles from epimastigotes using an iodixanol (Optiprep) density gradient (5). Short- and long-chain polyPs were concentrated towards the bottom (dense end) of the gradient (fractions 23-24), with a smaller peak close to the upper part of the gradient (fractions 5-9) (Fig. 2). Markers for other compartments all peaked further up the gradient in the region of fractions 5-9. As previously described, this middle peak also contains acidocalcisomes within ghosts of cells (26). Since the densest fractions (fractions 23 and 24) from the iodixanol gradients contained significant amounts (25% and 33%, respectively) of the total short- and long-chain polyPs recovered, which correlated well with the distribution of proton-translocating pyrophosphatase activity, an acidocalcisomal marker (4), the results suggest a preferential acidocalcisomal location of these compounds.

The location of polyP in T. cruzi was also investigated using 4´,6-diamidino-2-phenylindole (DAPI). DAPI is a useful tool in the fluorometric analysis of DNA but can also be used to study polyPs (27, 28). DAPI has a fluorescence emission maximum at 456 nm. PolyP shifts DAPI fluorescence to a higher wavelength with a maximum at about 525 nm (27). This DAPI fluorescence change is specific for polyP and is not produced by PPI or other anions (results not shown and 28). Epimastigotes of T. cruzi incubated in solutions of DAPI (10 µg/ml) were mounted on slides and examined by fluorescence microscopy. When a blue filter was used for DNA staining, the nuclei and kinetoplast were clearly visible (Fig. 3B). In contrast, when a red filter was used for polyP, staining in small spherical bodies corresponding to acidocalcisomes (3-6) was detected (Fig. 3A). No
staining was detected when DAPI was omitted and similar results were obtained using confocal microscopy (data not shown). To further confirm the acidocalcisomal localization of polyPs, acidocalcisomal fractions were incubated with DAPI, mounted on slides and examined by fluorescence microscopy. The isolated acidocalcisomes appeared in clusters and stained with DAPI when a red filter was used (Fig. 4). No fluorescence was detected when DAPI was omitted from the incubation medium or a blue filter was used (data not shown).

Changes in polyP levels during cell growth and differentiation- When T. cruzi epimastigotes were passaged into LIT medium, there was an initial lag period of 24-48 h before growth commenced. During this lag phase a rapid and massive accumulation of short- and long-chain polyP occurred (Fig. 5). Maximal accumulation of short-chain polyP was at about 12 h after inoculation and was followed by a rapid decrease at about 24 h. Maximal accumulation of long-chain polyP was at about 24 h and then rapidly decreased to steady state levels at about 72 h. Levels of both short- and long-chain polyP remained stable during the rest of the logarithmic and stationary phases of growth. These results suggest synthesis of short-chain polyP followed by their decrease simultaneously with the synthesis of long-chain polyP and finally hydrolysis of long-chain polyP once growth was resumed.

Since quantitative analysis of polyP indicated a larger amount of short- and long-chain polyP in amastigotes than in trypomastigotes, it was of interest to study how rapidly these changes occurred during differentiation. The transformation of T. cruzi trypomastigotes was induced by acidic medium (pH 5.0) and polyP content was
determined at different times. Short- (Fig. 6A) and long-chain (Fig. 6B) polyP synthesis was induced after only a two-hour incubation in the acidic medium (pH 5.0) and progressed with time. After overnight incubation, amastigote extracts contained amounts equivalent to those of amastigotes obtained from tissue cultures (Figs. 6A and 6B). Trypomastigotes in neutral pH (pH 7.5) medium did not show any significant increase in the amount of either short- or long-chain polyP at any time point (from time zero to overnight incubation) (Figs. 6A and 6B). A parallel transformation into amastigotes with an increase in the number of DAPI-positive vacuoles was also detected (data not shown). These results show that polyP synthesis occurs progressively during the development of amastigotes.

Changes in polyP levels under stress conditions- When submitted to hypo-osmotic stress, levels of short- (Fig. 7A) and long-chain (Fig. 7B) polyP decreased within 5-10 min. Long-chain polyP decreased to negligible levels after 20 min incubation under hypo-osmotic conditions (Fig. 7B). On the other hand, when epimastigotes were submitted to an hyperosmotic stress, short- (Fig. 7A) and long-chain (Fig. 7B) polyP levels increased within 5-10 min and remained stable until about 20 min. Similarly, epimastigotes submitted to an alkaline stress (incubation with 40 mM NH₄Cl) showed a progressive decrease in the levels of both short- (Fig. 8A) and long-chain (Fig. 8B) polyP.

Association of Ca²⁺ release and polyP hydrolysis- Hypo-osmotic and alkaline stresses have been shown before to result in increases in the intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) of different trypanosomatids (29-31), including *T. cruzi* (3). We
therefore investigated whether there was a correlation between Ca$^{2+}$ release from the acidic compartment containing most polyP (acidocalcisome) and polyP hydrolysis. In previous work (3) we showed that addition of nigericin (a H$^+$/K$^+$ exchanger), to epimastigotes previously exposed to ionomycin (a Ca$^{2+}$ ionophore) caused a secondary rise in [Ca$^{2+}$]$_i$ and that similar results were obtained when the order of additions was reversed. This indicated the existence of a Ca$^{2+}$ pool, in epimastigotes, that needed pH gradient neutralization for ionomycin-induced Ca$^{2+}$ transport to be effective. This is because ionomycin does not bind calcium below pH 7.0 (32) and cannot mobilize Ca$^{2+}$ out of acidic compartments. Using a similar protocol we observed that addition of ionomycin to epimastigotes previously exposed to nigericin resulted in significant decreases in short- (Fig. 8E) and long-chain (Fig. 8F) polyP and that similar results could be observed when the order of additions was reversed except that the nigericin effect was slower than the ionomycin effect (Figs. 8C and 8D). Taken together, the results in Fig. 8 suggest that processes that lead to alkalinization of the acidocalcisomes (NH$_4$Cl addition or treatment with ionophores) and result in [Ca$^{2+}$]$_i$ increase also result in polyP hydrolysis. Simultaneous measurement of changes in pH$_i$, [Ca$^{2+}$]$_i$, and short- and long-chain polyPs in amastigotes are shown in Fig. 9. Addition of ionomycin induced acidification of the cells and Ca$^{2+}$ release from intracellular compartments (EGTA was present in the extracellular medium to avoid Ca$^{2+}$ entry). This acidification was accompanied by immediate
hydrolysis of long chain polyP followed later by hydrolysis of short-chain polyP. Subsequent addition of nigericin resulted in a further acidification and Ca$^{2+}$ release accompanied by immediate hydrolysis of long-chain polyP and delayed hydrolysis of short-chain polyP.

_Synthesis and degradation of polyP in acidocalcisomes_- The rapid synthesis and hydrolysis of polyP that occurs during growth, differentiation and environmental stress of _T. cruzi_ suggested the presence of enzyme activities for polyP synthesis and degradation in the acidocalcisomes. In agreement with this suggestion, addition of ATP produced a significant increase in short- (SC) and long-chain (LC) polyPs in isolated acidocalcisomes within a few minutes (Fig. 10). This increase was time-dependent for at least 8 (LC) or 10 (SC) min (Figs. 10C and D) and depended on the previous acidification of the acidocalcisome produced by pre-incubation with PPi (Figs. 10A and B) suggesting a relationship between acidocalcisomal pH and polyP synthesis. This association has also been demonstrated in a _S. cerevisiae_ mutant defective in vacuolar H+-ATPase that fails to accumulate polyP in the vacuole (33). Evidence for a polyP hydrolyzing activity in the acidocalcisomes was also obtained. Fig. 11A shows that a significant increase in tetrapolyP hydrolysis was detected upon treatment of acidocalcisomes with 1 mg/ml digitonin. Supernatants isolated from acidocalcisomes treated with 1 mg/ml digitonin were shown to hydrolyze tetrapolyP at a rate of 0.38 ± 0.015 μmol/min x mg of protein at pH 7.5 (n = 22). This activity was lower at acidic pH (Fig. 11B) and was inhibited by high Ca$^{2+}$ concentrations (Fig. 11C), conditions that are prevalent within intact acidocalcisomes (4).
Interestingly a significant tetrapolyphosphatase activity was retained in the acidocalcisomal pellet after digitonin treatment. The rate of tetrapolyP hydrolysis in the acidocalcisomal pellet was $0.76 \pm 0.07 \mu$mol/min x mg of protein ($n = 9$) at pH 7.5.

**DISCUSSION**

In this study we have identified and measured the polyP content of different stages of *T. cruzi*. The results indicate the presence of high levels of short-chain polyP and lower levels of long-chain polyP. Analysis of purified *T. cruzi* acidocalcisomes, indicated that polyP was preferentially located in these organelles. This was confirmed by visualization of polyP in the acidocalcisomes using DAPI. The storage of phosphate as polyP appears ideal to reduce the osmotic effect of large pools of this crucial nutrient element. On the other hand its has the potential disadvantage that the cells need to be able to mobilize it under conditions of phosphate starvation or other forms of stress. Based on the total concentration of polyP in different stages of *T. cruzi* and the relative volume of acidocalcisomes in these cells (0.86%, 2.3%, and 0.26% of the total cell volume of epimastigotes, amastigotes and trypomastigotes, respectively (34)) and assuming that these compounds are essentially concentrated in acidocalcisomes, the calculated concentration in the organelles is in the molar range. Although polyPs could attain molar concentration in the acidic (pH 4-5) aqueous environment expected in the acidocalcisome, addition of divalent cations such as calcium or magnesium, present at stochiometric concentrations in the organelles (6), is expected to lead to almost quantitative precipitation of the resulting complexes. We thus
conclude that polyP in acidocalcisomes is most likely present as a microcrystalline aggregate. This conclusion is consistent with the very high electron density of acidocalcisomes in situ (6) and is further supported by the results of “magic angle” sample spinning (MASS) 31P-NMR experiments of intact parasites and acidocalcisomes (Moreno, Ruiz, Rodrigues, Bailey, Moreno, Urbina, Oldfield and Docampo, unpublished results). A high surface to volume ratio of the microcrystallites may be required for rapid metabolic turnover of polyPs accumulated in acidocalcisomes; other components of these organelles, such as carbohydrate (6) or lipids could be involved in maintaining this physical configuration.

A rapid increase (within 2-4 h) in the levels of short- and long-chain polyPs was detected during trypomastigote to amastigote differentiation and during the lag phase of growth of epimastigotes (within 12-24 h). Levels rapidly decreased after the epimastigotes resumed growth. These changes are different to those observed in bacteria and in yeast (35, 36). In bacteria, massive accumulations of polyP take place during the exponential phase of growth (35) and it has been proposed that polyP supports survival of stationary-phase E. coli (35). In S. cerevisiae it has been shown that, in glucose medium the mass and total cellular polyP content increased in parallel until glucose was depleted (11 h of culture growth) (36). After glucose depletion, the content of polyPs in the cells fell sharply and then increased again in a 24-h culture. The significant decline in the content of intracellular polyPs, while Pi was present in the growth medium at high concentrations, was suggested to imply that in this growth phase, polyP are an energy rather that phosphate source (36). Similarly, the changes observed in the content of polyP in T. cruzi epimastigotes and
amastigotes, which occur before cell division starts, could imply some requirement of these compounds as an energy source for resuming growth. It is important to note that the tissue culture-derived amastigotes assayed were either amastigotes released into the medium after 7 days of culture or after differentiation from trypomastigotes (Fig. 6). In both cases these amastigotes are in a lag phase of growth since they either did not start to divide (when differentiating from trypomastigotes) or they had already finished their intracellular division cycle and because of the fragility of the tissue culture cells were released without differentiating into trypomastigotes.

The presence of polyPs in various microorganisms is well established and the hypothetical roles of these molecules have been reviewed (10, 12, 36). The localization of these molecules within the cation-rich acidocalcisomes implies that their functional roles could be: (1) energy stores, and/or (2) chelators of metal ions. Short-chain polyPs such as PPI could be used in place of ATP as an energy donor in several reactions in trypanosomatids, such as the glycosomal pyruvate, phosphate dikinase (37) and the vacuolar-type proton-translocating pyrophosphatase that has been shown to drive proton uptake into the acidocalcisomal compartment through cleavage of cytosolic pyrophosphate (4). As PPI is a charged and polar molecule, any movement of PPI through a cell membrane is likely to involve a specialized channel or transporter. In this regard, a transmembrane transporter that shuttles PPI between intracellular and extracellular compartments has recently been identified in many mammalian tissues (38). A similar channel in the acidocalcisomal membrane would explain PPI accumulation after its synthesis through anabolic reactions occurring in the cytosol or its release to the cytosol to serve as substrate
for the V-H\(^+\)-PPase.

On the basis of the fast metabolic turnover of ATP (39) it has been suggested (40) that even highly elevated levels of long-chain polyP, that when expressed in phosphoanhydride bonds might be five times or more the level of ATP in some microorganisms, could supply energy for only a second or two. It has therefore been suggested that a regulatory role for long-chain polyP needs to be considered (40). Long chain polyP, even at relatively low levels, has been shown to be essential for adaptation to various stresses and for survival of bacteria in stationary phase (17, 35, 40). Similar studies have been reported in eukaryotic cells such as yeast (41, 42), fungi (43), and algae (44-46). In the yeast \(S.\) \textit{cerevisiae} and in the alga \textit{Dunaliella salina} ammonium ions induce hydrolysis of long-chain polyP and the appearance of tripolyP (41, 45). We have reported (47) that influx of ammonia into epimastigotes induces a rapid alkalinization of the cytoplasm followed by recovery of the cytoplasmic pH. This recovery occurs in parallel with massive hydrolysis of polyP (Figs. 8A, and B). In this regard, it has been indicated that \(H^+\) generation from polyP hydrolysis can neutralize up to a 2.5 pH unit change in \(S.\) \textit{cerevisiae} (42).

Two main classes of polyphosphatases have been described. Exopolyphosphatases have been found in prokaryotes and eukaryotes, and remove orthophosphate from the end of the polyphosphate chain. Although in bacteria these enzymes hydrolyse mostly high-molecular weight polyphosphates (48), at least some of the enzymes from yeast are more active hydrolyzing short-chain polyphosphates, such as tripolyphosphate (48). Endopolyphosphatases that act on long-chain polyP, generating tripolyphosphate, have
also been detected in eukaryotes, including the protist Giardia lamblia (49). Interestingly, the yeast endopolyphosphatase is localized in vacuoles (23). Enzymes that hydrolyze tripolyphosphate have also been reported from different organisms (50). Our results would be consistent with the presence of an exopolyphosphatase in acidocalcisomes that catalyzes the hydrolysis of long- and short-chain polyPs to Pi. This exopolyphosphatase is probably tightly associated to the acidocalcisomal membrane as a high activity is still retained in the membrane fraction after detergent treatment of the organelles. It is intriguing that a gene with homology to exopolyphosphatases has recently been found in L. major (51), providing the first evidence for a breakdown pathway for these molecules in trypanosomatids. Our results would also suggest the presence of polyphosphate kinase in acidocalcisomes. Organelle acidification and addition of ATP were necessary to detect polyP synthesis. This would suggest that either the acidocalcisomal membrane is permeable to ATP or the enzyme is located in the acidocalcisomal membrane with its catalytic site oriented towards the cytosolic side of the organelle, and requires an intraorganellar acidic pH for activity.

Short- and long-chain polyP levels also rapidly decreased upon exposure of epimastigotes to hypo-osmotic stress while levels increased after hyper-osmotic stress (Fig. 7A and B). This would suggest a role for Pi in the adaptation of the parasites to osmotic stress. This is extremely important for a parasite that lives in environments of widely different osmotic conditions such as the intestine of the insect vector, the bloodstream and the cytosol of host cells. A role for acidocalcisomes in the response of L. major promastigotes to osmotic stress has recently been proposed on the basis of their changes in Na and Cl content after hypo-osmotic stress (52).
Ca²⁺ release from acidocalcisomes by a combination of ionophores (ionomycin, nigericin) was associated with short- and long-chain polyP hydrolysis. Ionomycin is not effective in releasing Ca²⁺ from acidic compartments (32). However, acidification of the cytosol (Fig. 9A) could provide some driving force for Ca²⁺ release through a Ca²⁺/H⁺ exchanger, the presence of which has been demonstrated in trypanosomatid acidocalcisomes (53). Further addition of nigericin leads to alkalinization of the acidocalcisomes by K⁺/H⁺ exchange and further acidification of the cytosol, which would favor further Ca²⁺ release (Fig. 9A). Release of Ca²⁺ and alkalinization of the acidocalcisomes would result in activation of the polyP hydrolyzing activities in the organelles.

It is also currently hypothesized that one of the main roles of the acidocalcisome in T. cruzi is calcium storage for use in intracellular signaling, particularly in the infective stages (2). Enzymes cleaving short- and long-chain polyPs to orthophosphate in acidocalcisomes may therefore indirectly regulate intracellular calcium content. In this regard an endogenous Ca²⁺-inhibited pyrophosphatase activity was postulated to be involved in PPI hydrolysis in the volutin granules of Tetrahymena pyriformis (54).

In conclusion, our results indicate that the concentrations of polyP change drastically during growth and differentiation of T. cruzi and that they are rapidly mobilized under osmotic or alkaline stresses. Ca²⁺ release from acidocalcisomes is associated with the acidocalcisomal hydrolysis of polyP. The rapid mobilization of Ca²⁺ and polyP from
acidocalcisomes suggests a critical role for these organelles in the adaptation of the parasite to environmental changes.

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REFERENCES


8. Urbina, J. A., Moreno, B., Vierkotter, S., Oldfield, E., Payares, G., Sanoja, C., Bailey, B. N., Yan,


Table I: Distribution of polyP in different fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>SC PolyP (µmoles)</th>
<th>LC PolyP (µmoles)</th>
<th>Total protein (mg)</th>
<th>Purification -fold (SC/LC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate</td>
<td>10.08 ± 3.30</td>
<td>0.17 ± 0.07</td>
<td>147 ± 7.8</td>
<td>1/1</td>
</tr>
<tr>
<td>10,000g supernatant</td>
<td>0.54 ± 0.25</td>
<td>0.007 ± 0.003</td>
<td>60 ± 7.3</td>
<td>0.15/0.1</td>
</tr>
<tr>
<td>10,000g pellet</td>
<td>9.93 ± 0.04</td>
<td>0.16 ± 0.02</td>
<td>36 ± 1.5</td>
<td>4.5/3.93</td>
</tr>
<tr>
<td>Isolated Acidocalcisomes</td>
<td>1.69 ± 0.10</td>
<td>0.074 ± 0.002</td>
<td>0.56 ± 0.02</td>
<td>50/114</td>
</tr>
</tbody>
</table>

SC/LC, short-chain and long-chain polyP
Figure legends:

FIG. 1. Urea-PAGE analysis of polyP from different stages of *T. cruzi*. PolyP extracted from epimastigotes (Epis), trypomastigotes (Tryp), and amastigotes (Amas) was electrophoresed by 6% urea-PAGE. Chain lengths of standards are on the left. The position of migration of long-chain polyP is indicated with an arrow. B, Incorporation of $[^{32}\text{P}]\text{Pi}$ into short- and long-chain polyP in epimastigotes. Cells ($1 \times 10^9$ epimastigotes) were incubated for 60 min in medium supplemented with $[^{32}\text{P}]\text{Pi}$. The polyPs were extracted and analyzed by Urea-PAGE. Lane 1 is with 0.2% and lane 2 with 8% of the incorporated $[^{32}\text{P}]\text{Pi}$.

FIG. 2. Distribution of short- (SC) and long-chain (LC) polyPs from epimastigotes on iodixanol gradients. PolyPs are concentrated in a distinct dense fraction. PolyP content was compared with the distribution of established organelle markers, hexokinase (glycosome), acid phosphatase (lysosome), alanine (ALT) and aspartate aminotransferases (AST) (mitochondria and cytosol) and vacuolar pyrophosphatase (acidocalcisome).

FIG. 3. Accumulation of DAPI in acidocalcisomes of epimastigotes of *T. cruzi*. Cells were treated with DAPI for 10 min and observed with a red filter for polyP detection (A) or a blue filter (B) for nucleic acid detection. C, is a bright field micrograph of the same cell. Note the numerous fluorescent vacuoles (acidocalcisomes) in A, and the nucleus and kinetoplast in B. Bar, 10 µm.
FIG. 4. Accumulation of DAPI in isolated acidocalcisomes. Acidocalcisomes were isolated and treated with DAPI as described under Experimental Procedures. A, acidocalcisomes observed with a red filter for polyP detection. B, bright field micrograph of the same fraction. Bar, 10 µm.

FIG. 5. PolyP accumulation during growth of epimastigotes. Short- (open squares) and long-chain (closed squares) polyPs accumulate during the lag phase of growth (closed triangles).

FIG. 6. PolyP increase during trypomastigote to amastigote transformation. T. cruzi trypomastigotes were incubated at pH 5.0 (closed bars) or pH 7.5 (open bars) for 0, 2, 4, and 12 h and short-chain (A) and long-chain (B) polyP were quantified as described under Experimental Procedures. The average ± S.D. from 3 experiments are shown. The cellular content of short- and long-chain polyP did not vary significantly after 2, 4, and 12 h at pH 7.5, but increased significantly after 2, 4, and 12 h at pH 5.0 with $P < 0.05$ as determined by the Student’s $t$ test.

FIG. 7. Effect of osmotic shock on the polyP content of epimastigotes. Epimastigotes were resuspended in isotonic (300 ± 5 mosM, closed diamonds), hypo-osmotic (150 ± 4 mosM, closed squares) or hyperosmotic (450 ± 6 mosM, open squares) medium, as described under Experimental Procedures. At the indicated times, aliquots were withdrawn, quickly transferred to an isotonic buffer pre-equilibrated at 4°C, to stop the reaction, centrifuged,
and the polyP content of the pellets was quantified as described under Experimental Procedures. A, shows changes in short-chain polyP and B, changes in long-chain polyP.

FIG. 8. **The effect of alkaline stress or ionophores on the polyP content of epimastigotes.**

Epimastigotes (2.5 x 10⁸) were washed once with Dulbecco’s PBS and resuspended in 0.55 ml 116 mM NaCl, 5.4 mM KCl, 0.8 mM Mg SO₄, 5.5 mM glucose, 1 mM EGTA, 50 mM Tris-HCl pH 7.4. At the times indicated 40 mM NH₄Cl, 1 µM ionomycin or 1 µM nigericin were added and short-chain (A, C, E) or long-chain (B, D, F) polyP content was determined as indicated under Experimental Procedures. Dashed lines indicate second addition. Data are of single representative experiments with data points given as means ± SEM.

FIG. 9. **The effect of ionophores on the intracellular calcium concentration, intracellular pH and polyP content of T. cruzi amastigotes.** Amastigotes were loaded with fura 2/AM or BCECF/AM and suspended in 116 mM NaCl, 5.4 mM KCl, 0.8 mM Mg SO₄, 5.5 mM glucose, 1mM EGTA and 50 mM Tris pH 7.4. At the times indicated 1 µM ionomycin or 1 µM nigericin were added. Intracellular calcium (broad line) and pH (narrow line) changes were determined as indicated under Experimental Procedures (A). In parallel experiments short- (B) and long-chain (C) polyP content of the samples was examined as described under Experimental Procedures. Closed squares are controls with no additions. Open squares are after addition of ionomycin and closed circles are after addition of nigericin. Data depicted in panels B and C are of single representative experiments with data points given as means ± SEM.
FIG. 10. PolyP synthesis in acidocalcisomes: In A and B isolated acidocalcisomes were resuspended in buffer A and incubated for 5 min with or without 0.1 mM PPI. Then, 1 mM ATP (lane 3 and 7) or buffer A (lane 4 and 8) was added. After 5 min at 37 °C, long-chain (LC, A) and short-chain (SC, B) polyP were extracted and quantified as described under Experimental Procedures. C and D show the time-dependent accumulation of long-chain (C) and short-chain (D) polyP in isolated acidocalcisomes after addition of ATP to the samples preincubated with 0.1 mM PPI for 5 min. Data are from a single representative experiments with data points given as mean ± SEM.

FIG. 11. Tetrapolyphosphatase activity released from acidocalcisomes upon treatment with 1 mg/ml digitonin. A, Tetrapolyphosphatase activity of acidocalcisomal pellets treated or untreated with 1 mg/ml digitonin (n = 5). B, Range of pHs of tetrapolyphosphatase activity present in the supernatant fraction of digitonin-treated acidocalcisomes (n = 6). C, Dose-dependent inhibition of tetrapolyphosphatase activity present in the supernatant fraction of digitonin-treated acidocalcisomes, by CaCl₂ (n = 7).
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Figure 2, Ruiz et al.
Figure 6, Ruiz et al.
Figure 7, Ruiz et al.
Figure 8, Ruiz et al.
Figure 9. Ruiz et al.
Figure 11, Ruiz et al.
Rapid changes in polyphosphate content within acidocalcisomes in response to cell growth, differentiation and environmental stress in Trypanosoma cruzi
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