Review

Evolution of acidocalcisomes and their role in polyphosphate storage and osmoregulation in eukaryotic microbes

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Acidocalcisomes are acidic electron-dense organelles, rich in polyphosphate (poly P) complexed with calcium and other cations. While its matrix contains enzymes related to poly P metabolism, the membrane of the acidocalcisomes has a number of pumps (Ca²⁺-ATPase, V-H⁺-ATPase, H⁺-PPase), exchangers (Na⁺/H⁺, Ca²⁺/H⁺), and at least one channel (aquaporin). Acidocalcisomes are present in both prokaryotes and eukaryotes and are an important storage of cations and phosphorus. They also play an important role in osmoregulation and interact with the contractile vacuole complex in a number of eukaryotic microbes. Acidocalcisomes resemble lysosome-related organelles (LRO) from mammalian cells in many of their properties. They share similar morphological characteristics, acidic properties, phosphorus contents and a system for targeting of their membrane proteins through adaptor complex-3 (AP-3). Storage of phosphate and cations may represent the ancestral physiological function of acidocalcisomes, with cation and pH homeostasis and osmoregulatory functions derived following the divergence of prokaryotes and eukaryotes.

Keywords: acidocalcisome; calcium; polyphosphate; pyrophosphate; volutin granules; protists

1. INTRODUCTION

One of the first subcellular structures recognized in bacteria was the metachromatic (Babes 1895) or volutin (Meyer 1904) granule. The name volutin granule derives from their discovery in the bacterium Spirillum volutans, in which the granules stain red when treated with toluidine blue. The presence of these granules was used as a diagnostic feature of important bacteria such as Corynebacterium diphtheriae (Kornberg 1995). Over the years, volutin granules have also been described in lower eukaryotes such as algae, yeasts and protozoa.

Volutin granules were also found in a number of eukaryotic microbes using the ‘Meyer test’ based on their methachromasy, including coccidia (Kunze 1907), trypanosomes (Swellengrebel 1908) and Sarcosporidia (Erdmann 1910). More recently, elemental analysis of these granules in different trypanosomatids revealed the presence of large amounts of phosphorus as well as calcium and other cations (Vickerman & Tetley 1977; Dvorak et al. 1988; LeFurgey et al. 1990). These granules were later identified (Scott et al. 1997) as the acidic, calcium-rich compartments of trypanosomes known as acidocalcisomes (Vercesi et al. 1994; Docampo et al. 1995).

Acidocalcisomes can thus be defined as electron-dense acid organelles with a high concentration of phosphorus present as poly P complexed with calcium and other elements (Docampo et al. 2005). The identification of enzymes and transporters in the surrounding membranes of these granules in prokaryotes (Seufferheld et al. 2003, 2004) and eukaryotes (Docampo & Moreno 1999) established them as real organelles. The dense granules of human platelets possess similar characteristics to acidocalcisomes (Ruiz et al. 2004), suggesting that the organelles play important roles that may have been conserved following the divergence of prokaryotes and eukaryotes.

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Alternatively, the presence of acidocalcisomes in both prokaryotes and eukaryotes could be an example of convergent evolution.

Acidocalcisomal poly P stores are important for resistance to heavy metals in some eukaryotes and prokaryotes (Hashemi et al. 1994; Alvarez & Jerez 2004; Andrade et al. 2004; Remonsellez et al. 2006; Nagasaka & Yoshimura 2008). Thus, phosphate and heavy metal storage may represent an ancestral characteristic that was important for their evolutionary development. Phosphate storage is especially evident in marine plankton, in which nearly 10 per cent of the phosphorus pools are composed of poly P (Diaz et al. 2008), a reserve that some algae actively manage to overcome phosphate limitation in phosphate-poor water (Watanabe et al. 1988).

Acidocalcisomal poly P complexes protons in addition to sequestering heavy metals in eukaryotes, linking poly P inextricably to pH homeostasis. Poly P stores are inversely related to acidity in a wide variety of lower eukaryotes including Candida humicola (McGrath & Quinn 2000), Dunaliella salina (Pick & Weiss 1991) and trypanosomatids (Ruiz et al. 2001a; Lemercier et al. 2002; Rohloff & Docampo 2006). However, regulation of pH by acidocalcisomal poly P appears restricted to eukaryotes, suggesting that this characteristic was derived after divergence of the eukaryotic and prokaryotic lineages.

**2. STRUCTURAL CHARACTERISTICS AND COMPOSITION OF ACIDOCA LCSOMES**

Acidocalcisomes of bacteria and eukaryotic microbes can be easily identified with dyes that accumulate into acidic compartments, such as acridine orange (Vercesi et al. 1994; Docampo et al. 1995; Miranda et al. 2008) and LysoSensor blue DND-167 (Seufferheld et al. 2003), and dyes that stain poly P, such as 4′-6′-diamino-2-phenylindole (DAPI; Scott & Docampo 2000; figure 1a). They typically are spherical with an average diameter of 0.2–0.5 μm although polymorphic morphologies occur in some cells (Docampo et al. 2005). Their position in the cells is random.

By transmission electron microscopy the organelle appears empty or with an inclusion of a thin layer of dense material that sticks to the inner face of the membrane (figure 2). While standard electron microscopy protocols extract electron dense material, acidocalcisomes can be directly observed with whole mounts of cells, bacteria or protists deposited onto carbon- and formvar-coated grids. They appear as electron-dense spheres (figure 3).

The elemental composition of the organelle has been analysed by electron microscopy techniques (X-ray microanalysis and elemental mapping) and has invariably revealed the presence of large deposits of phosphorus and calcium. Other cations (magnesium, sodium, potassium, zinc, iron) occur in lower amounts depending on the cell analysed (Docampo et al. 2005).

Acidocalcisomes from bacteria and eukaryotic microbes are very rich in pyrophosphate (PPi) as well as in short-chain (fewer than 50 Pi units) and long-chain (50–800 Pi units) poly P. The concentration of poly P within these granules could reach molar levels (in terms of Pi units; Docampo et al. 2005), probably explaining their high electron density. In fact, solid-state condensed phosphates can be detected in isolated acidocalcisomes by magic-angle spinning NMR techniques (Moreno et al. 2002). Acidocalcisomes are enriched in basic amino acids that probably complex with the negatively charged poly P (Rohloff et al. 2003). Only a few soluble enzymes have been detected in the acidocalcisomal matrix, including an exopolyphosphatase (PPX) in Leishmania.
Acidocalcisomes are similar to the plant vacuole in that they possess a number of pumps, channels and cation exchangers in their membranes. Both the plant vacuole and acidosomes have two proton pumps: a vacuolar-type H\(^+\)-ATPase (V-H\(^+\)-ATPase; Lu et al. 1998) and a vacuolar proton translocating pyrophosphatase (V-H\(^+\)-PPase; Docampo et al. 2005; figure 1c), which is absent in animals and fungi. Coexistence of different proton pumps in the membrane or tonoplast of the plant vacuole may play a role in energy conservation (Rocha-Façanha & de Meis 1998). The H\(^+\) gradient generated across the plant vacuolar membrane by the hydrolysis of either PP\(i\) or ATP may drive both ATP and PP\(i\) synthesis by reversal of the tonoplast H\(^+\)-ATPase (Rocha-Façanha & de Meis 1998; Hirata et al. 2000) or the V-H\(^+\)-PPase (Rocha-Façanha & de Meis 1998), and a similar process could occur in acidocalcisomes. However, both pumps do not co-localize in all acidocalcisomes. Ruiz et al. (2001b) used antibodies against these two proteins in Chlamydomonas reinhardtii to demonstrate the presence of different populations of organelles, some containing both proteins and others containing only a single pump type. Similarly, co-localization studies of the V-H\(^+\)-PPase with a Ca\(^{2+}\)-ATPase in T. cruzi revealed two apparently different populations of acidocalcisomes (Lu et al. 1998). Physiological experiments in L. donovani also suggested the presence of the V-H\(^+\)-ATPase and the V-H\(^+\)-PPase in different compartments (Rodrigues et al. 1999b).

The V-H\(^+\)-PPase also exists elsewhere in some species. It was initially described in chromatophore membranes of Rhodospirillum rubrum (Baltscheffsky et al. 1966; Moyle et al. 1972) and in plant vacuoles (Rea & Poole 1986), but it has also been detected in the plasma membrane and the Golgi complex of some plants (Long et al. 1995; Robinson et al. 1996) and T. cruzi (Scott et al. 1998; Martinez et al. 2002). The V-H\(^+\)-PPase of Toxoplasma gondii has been found in a vacuolar compartment involved in microneme protein maturation (Harper et al. 2006), while the Plasmodium spp. enzyme has been found in the acidocalcisomes (Luo et al. 1999; Marchesini et al. 2000), digestive vacuole (Saliba et al. 2003) and plasma membrane (McIntosh et al. 2001).

There is also evidence for the presence of a Ca\(^{2+}\)-ATPase in acidocalcisomes from several eukaryotic microbes, such as T. cruzi (Scott & Docampo 2000), T. brucei (Rodrigues et al. 1999a; Luo et al. 2004), Dictyostelium discoideum (Marchesini et al. 2002) and T. gondii (TyAl; Luo et al. 2001; Luo et al. 2005).

Na\(^+/H^+\) and Ca\(^{2+}/H^+\) exchanger activities have been detected in acidocalcisomes of T. brucei procyclic forms (Vercesi & Docampo 1996; Vercesi et al. 1997) and in L. donovani promastigotes (Vercesi et al. 2000). Finally, an aquaporin has also been identified in acidocalcisomes of T. cruzi (Montalvetti et al. 2004). The protein acts as a water channel and is unable to transport glycerol when expressed in Xenopus oocytes. This aquaporin is also localized to the contractile vacuole complex, suggesting a role in osmoregulation (Rohloff et al. 2004). Figure 4 shows a scheme of the
known components of acidocalcisomes in early eukaryotes.

4. ACIDOCALCISOME ENZYMES INVOLVED IN POLYPHOSPHATE SYNTHESIS AND DEGRADATION

(a) Poly P biosynthesis

Biosynthetic enzymes for poly P were largely uncharacterized in eukaryotes until recently. A poly P kinase (PPK) homologous to prokaryotic PPK1 was reported in just a single eukaryote, D. discoideum (DdPPK1, Zhang et al. 2007), and proposed to be a product of horizontal gene transfer (Hooley et al. 2008). It is present in small vesicles, which may correspond to acidocalcisomes (Marchesini et al. 2002; Zhang et al. 2007), although no co-localization studies have been reported. DdPPK1 catalyses the following reaction:

$$\text{Poly}P_n + \text{ATP} \rightarrow \text{Poly}P_{n+1} + \text{ADP}. \quad (4.1)$$

A second PPK, termed DdPPK2, which catalyses the same reaction, was proposed to be present in acidocalcisomes of D. discoideum (Gomez-Garcia & Kornberg 2004). DdPPK2 shares characteristic and sequence identity with actin-related proteins, a group of proteins with homology to muscle actins. Actin inhibitors such as phalloidin and DNase I inhibited DdPPK2-mediated synthesis of poly P. This particular actin-related protein complex can polymerize into an actin-like filament concurrent with its synthesis of a poly P chain in a fully reversible reaction (Gomez-Garcia & Kornberg 2004). The presence of a DdPPK2-like activity in C. reinhardtii was also reported (Gomez-Garcia & Kornberg 2004), and an unidentified PPK activity was also detected in acidocalcisomes of T. cruzi (Ruiz et al. 2001a).

Using DNA microarray methodology, Ogawa et al. (2000) identified four PHM genes in Saccharomyces cerevisiae that encode proteins involved in poly P synthesis as shown by the lack of detectable poly P in phm3Δ and phm4Δ mutants or in phm1Δ–phm2Δ double mutants. These authors proposed that the protein products of these genes are poly P synthases (Ogawa et al. 2000). Since then protein sequence homologues from several organisms have been annotated in genome databases as poly P synthases. The PHM genes were independently identified by Cohen et al. (1999) and named vacuolar transporter chaperone (VTC) 1–4 (VTC1/PHM4, VTC2/PHM1, VTC3/PHM2, and VTC4/PHM3; Cohen et al. 1999; Nelson et al. 2000). A protein (TbVTC1) homologous to the yeast vacuolar transporter chaperone 1 (Vtc1p) was identified in T. brucei that was essential for poly P synthesis, acidocalcisome biogenesis and cytokinesis.

Figure 3. Morphology of trypanosomatid acidocalcisomes. Electron spectroscopic imaging (contrast tuning) of whole cells (a–c) or fractions (d) adhered to formvar-coated grids showing the shape, size and distribution of acidocalcisomes (black spots) in different species. (a) Blastochitidia culicis; scale bar, 2 μm. (b) Herpetomonas angusteri; scale bar, 3 μm. (c) Phytomonas serpens; scale bar, 0.5 μm. (d) Isolated acidocalcisomes from Trypanosoma cruzi; scale bar, 0.5 μm. Note the polymorphic nature of acidocalcisomes in (c). b,c are adapted from Miranda et al. (2004, Copyright Elsevier).
TbVTC1 was shown to co-localize with the vacuolar V-H\textsuperscript{+}-PPase to the acidocalcisomes. RNA interference experiments altered acidocalcisomal morphology and significantly decreased the amount of poly P (Fang et al. 2007a).

Many apicomplexan and trypanosomatid parasite genomes include sequences with homology to Phm/Vtc proteins (Fang et al. 2007a). Analysis of these sequences revealed that there are both small and large proteins with Phm/Vtc homology. Large homologues (66.1–129.0 kDa) were detected in *Saccharomyces pombe*, *Candida albicans*, *Encephalitozoon cuniculi*, *T. gondii*, *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium falciparum*, *L. major*, *T. brucei* and *T. cruzi*. Short homologues (13.4–19.9 kDa) to *S. cerevisiae* Phm4p/Vtc1 were detected in *S. pombe*, *T. cruzi*, *T. brucei*, and *L. major*. Regardless of size, all proteins examined shared a conserved motif, located centrally (*T. brucei*, *T. cruzi*, *L. major*) or near the N-terminus (*S. cerevisiae*, *S. pombe*) in the case of the short Phm4p/Vtc1 homologues and near the C-terminus in the case of the long homologues. Most of these sequences have not been experimentally examined (Fang et al. 2007a). The recent identification of the Phm/Vtc family as a poly P polymerase and translocase complex in *S. cerevisiae* (Hothorn et al. 2009) suggests that this complex is involved in the synthesis of acidocalcisomal poly P in most eukaryotic microbes. ScVtc4 was identified as the catalytic subunit of the complex (Hothorn et al. 2009), thus explaining the conservation of this subunit in other fungi as well as in protists, which usually have only two of the four subunits present in yeast. Vtc4 also possesses an SPX domain. SPX domains are usually at the N-termini of proteins and are thought to have a regulatory function (Hürlimann et al. 2009). The VTC complex has been found only in marine organisms (diatoms), fungi and protists, but appears not to be conserved in animals or plants. It is not known whether all the organisms possessing the VTC complex possess acidocalcisomes, although structures closely resembling acidocalcisomes have also been found in fungi (Franzen et al. 2008).

**Poly P hydrolysis**

Degradation of poly P in eukaryotic microbes is catalysed by PPXs and endopolyphosphatas (PPNs), but only a PPX (in *L. major*, LmPPX) has been
detected in acidocalcisomes (Rodrigues et al. 2002). The enzyme also localizes to the cytosol (Rodrigues et al. 2002) and catalyses the following reaction:

\[
\text{PolyP}_n + (n-2)\text{H}_2\text{O} \rightarrow (n-2)\text{Pi} + \text{PPi}. \quad (4.2)
\]

PPX progressively hydrolyses poly P from the chain termini producing Pi until only PPi remains. Recombinant LmPPX is similar to yeast PPX (ScPPX, Wurst et al. 1995) with respect to its Mg\(^{2+}\) requirement, optimum pH, and sensitivity to cations, amino acids and heparin. In contrast to the yeast enzyme and other PPXs, LmPPX degrades short chain poly P with higher rates and affinity. The \(T.\) \(cruzi\) PPX is similar to the LmPPX although its localization has not been reported. Interestingly, overexpression of TcPPX led to a significant decrease in short-chain poly P and in the staining of acidocalcisomes with DAPI, suggesting that it is also localized to acidocalcisomes (Fang et al. 2007b).

Acidocalciosomal soluble vacuolar pyrophosphatases (VSP) have also been described in \(T.\) \(brucei\) (Lemercier et al. 2004) and \(L.\) \(amazonsensis\) (Espiau et al. 2006). VSP require the presence of transition metal ions such as Zn\(^{2+}\), Mn\(^{2+}\) and Co\(^{2+}\) to hydrolyse poly P, a property that they share with the homologue \(S.\) \(cerevisiae\) pyrophosphatase (Oksanen et al. 2007). This could be physiologically important as acidocalciosmes of these parasites are rich in Zn\(^{2+}\).

5. OSMOREGULATORY FUNCTIONS OF ACIDOCALCISOMES AND RELATION TO THE CONTRACTILE VACUOLE COMPLEX

In addition to their function as storage organelles for phosphorus and cations, acidocalcisomes appear to have an important role in osmoregulation.

The contractile vacuole is an organelle involved in osmoregulation in a number of free living and parasitic protists and has a bipartite structure consisting of a central vacuole, or bladder, and a surrounding network of microtubules and vesicles named the spongome (Bowers & Korn 1968; figure 5). Early work demonstrated that acidocalcisomes are in close contact with the contractile vacuole of \(D.\) \(discoideum\) (Marchesini et al. 2002). Submission of \(D.\) \(discoideum\) amoebas to hypsometric shock increased this association. In addition to poly P, both compartments possess a \(V-H^+\)-ATPase, a \(\text{Ca}^{2+}\)-ATPase and a \(H^+\)-PPase. Marchesini et al. (2002) proposed that poly P hydrolysis could lead to water uptake by the vacuole, thereby contributing to volume regulation under hypometric stress. Contractile vacuoles of \(C.\) \(reinhardtii\) are also rich in poly P, and also have a \(V-H^+\)-ATPase and a \(V-H^+\)-PPase (Ruiz et al. 2001b).

A more detailed study of the association of acidocalcisomes and the contractile vacuole complex was performed in trypanosomatids. When \(T.\) \(cruzi\) epimastigotes are exposed to hypometric or hyperosmotic stress conditions, there is a rapid hydrolysis or synthesis of acidocalciosomal poly P, respectively (Ruiz et al. 2001a), suggesting a link between acidocalcisomes and osmotic homeostasis. In addition, exposure of \(L.\) \(major\) promastigotes to hypometric stress alters sodium and chloride content of their acidocalcisomes, implicating their role in this response (LeFurgey et al. 2001). In \(T.\) \(cruzi\), an aquaporin or water channel (TcAQPI) is located in both acidocalcisomes and the contractile vacuole complex (Montalvetti et al. 2004). Hyposometric stress leads to an increase in cyclic adenosine monophosphate (AMP), which stimulates translocation of TcAQPI from the acidocalcisome to the contractile vacuole. This translocation probably results in water movement leading to a decrease in cell volume (Rohloff et al. 2004). Additional evidence for a role of acidocalcisomes in osmoregulation resulted from studies on \(T.\) \(brucei\) (Lemercier et al. 2004; Fang et al. 2007b). The use of RNAi to reduce the expression of the acidocalcisomal soluble pyrophosphatase (TbVSP1) resulted in trypanosomes that were deficient in poly P
and in their response to hyposmotic stress (Lemercier et al. 2004). Ablation of a vacuolar transporter chaperone (VTC1) in *T. brucei* by RNAi resulted in abnormal morphology of acidocalcisomes, decrease in cellular poly P content, and a deficient response to hyposmotic stress (Fang et al. 2007a).

In addition to the cyclic AMP pathway, other signalling systems have been found to be involved in osmoregulation in *T. cruzi*. Overexpression of a phosphatidylinositol 3-kinase (PI3K, or Vps34) in *T. cruzi* resulted in morphological and functional alterations related to vesicular trafficking, and the cells were more resistant to hyposmotic stress (Schoijet et al. 2008). Interestingly, these cells had large contractile vacuole bladders (Schoijet et al. 2008).

6. RELATION OF ACIDOCALCISOMES TO LYSOSOME-RELATED ORGANELLES

Recent work has indicated that acidocalcisomes share characteristics with organelles known as lysosome-related organelles (LROs) and may be biogenetically related. LROs comprise a heterogeneous set, many of which are secreted from the cell (Cutler 2002). Examples include melanosomes, lytic granules, major histocompatibility complex II compartments, platelet dense granules, basophil granules and neutrophil azurophil granules (Dell’Angelica et al. 2000). Acidocalcisomes resemble LROs in many respects. For example, one type of LRO, platelet dense granules, have a similar size, high electron density, are acidic, and contain calcium and phosphorus in the form of poly P and PPI (Ruiz et al. 2004).

While endocytic tracers (transferrin, Scott et al. 1997; horseradish peroxidase, Coppons et al. 1993; FM4-64, Mullin et al. 2001) do not accumulate in acidocalcisomes, some accumulation of endocytic markers occur when *T. cruzi* is treated with an inhibitor of the sterol biosynthetic pathway (Vannier-Santos et al. 1999). In *L. major*, a mutant deficient in sphingolipid synthesis was found to be defective in biogenesis of both multivesicular bodies (or late endosomes) and acidocalcisomes, suggesting that these compartments have a common origin (Zhang et al. 2005). Besteiro et al. (2008) recently found that adapter protein 3 (AP-3), a protein involved in transport of membrane proteins to lysosomes and LROs in other cells, has a similar function with respect to acidocalcisomes in *L. major*, providing support for a close similarity between acidocalcisomes and the endo/lysosomal system. Furthermore, mutants of *T. brucei* deficient in an orthologue of vacuolar sorting protein 41 (VSP41p), which interacts with the δ subunit of AP-3–coated carrier vesicles (Rehling et al. 1999) and is involved in the biogenesis of LROs (Dell’Angelica et al. 2000), had large numbers of small intracellular vesicles similar to acidocalcisomes (Lu et al. 2007). The finding that LROs and acidocalcisomes share the system for targeting of their membrane proteins reinforces the similarities between these organelles (Besteiro et al. 2008), supporting the hypothesis that LROs and acidocalcisomes are biogenically related.

7. CONCLUSION

Acidocalcisomes were known for many years as volutin or poly P granules and are present in both prokaryotes and eukaryotes. They are related to a group of eukaryotic organelles known as LROs. We know that acidocalcisomes are important storage compartments for phosphorus and cations as well as basic amino acids in some cells. Their acidity is maintained by proton pumps, one of which, a V-H⁺-PPase, is only present in bacteria, plants, and early divergent eukaryotes. In addition to pumps, exchangers and aquaporin, acidocalcisomes possess several enzymes involved in PPI and poly P metabolism. Some of these enzymes, such as DdPPK2, have not yet been found in other organisms while the vacuolar transporter chaperone complex is only present in eukaryotic microbes. Many of the acidocalcisome enzymes are unique to different microbes and are therefore potential targets for new drugs, as we noted in a recent review (Docampo & Moreno 2008). Acidocalcisomes play an important role in response to osmotic stress, and their interactions with the contractile vacuole complex of free living and parasitic organisms are very relevant to this function. Many things are not yet known about acidocalcisomes, such as their biogenesis, the phylogenetic relationships of their various enzymes, the mechanism for accumulation and release of their phosphate and cationic components, and the functions of the components accumulated in their matrix. This is an exciting area of work and many novel functions of poly P and acidocalcisomes await discovery.

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