AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology and Molecular Biology Reviews

Eukaryotic Cells | Review

# Advances in the cellular biology, biochemistry, and molecular biology of acidocalcisomes

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**SUMMARY** Acidocalcisomes are organelles conserved during evolution and closely related to the so-called volutin granules of bacteria and archaea, to the acidocalcisome-like vacuoles of yeasts, and to the lysosome-related organelles of animal species. All these organelles have in common their acidity and high content of polyphosphate and calcium. They are characterized by a variety of functions from storage of phosphorus and calcium to roles in $Ca^{2+}$ signaling, osmoregulation, blood coagulation, and inflammation. They interact with other organelles through membrane contact sites or by fusion, and have several enzymes, pumps, transporters, and channels.

**KEYWORDS** acidocalcisome, blood clotting, calcium, dense granules, polyphosphate

## INTRODUCTION

Acidocalcisomes (ACs) are specialized organelles found in a variety of organisms and characterized by their acidic pH and high concentrations of calcium and polyphosphate (polyP). Initially identified in trypanosomatids, they have been related to the metachromatic or volutin granules, first reported in bacteria (1), and to the lysosome-related organelles (LROs) present in animal cells (2). These connections suggest that

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The author declares no conflict of interest.

See the funding table on p. 28.

**Published** 15 December 2023

storage of polyP and calcium in acidocalcisomes or acidocalcisome-like organelles is a conserved property of both prokaryotes and eukaryotes.

A combination of microscopy techniques and biochemical analyses was initially used to identify and characterize these organelles in trypanosomatid parasites (3, 4). These were found to have a low pH sustained by a bafilomycin-sensitive proton pump (V-$H^+$-ATPase), and high calcium, which was taken up by a P-type $Ca^{2+}$-ATPase (4). X-ray microanalyses (5) showed that the organelles corresponded to the "inclusion vacuoles" (6) or "dense granules" (7, 8), previously identified to possess large quantities of phosphorus and cations in different trypanosomatids. Later work found that another proton pump, a vacuolar proton pyrophosphatase, V-$H^+$-PPase, was also present in these trypanosomatid organelles (9) and could be used as a suitable marker for their isolation (10). $^{31}P$ nuclear magnetic resonance (NMR) (11, 12) and biochemical analyses (13) uncovered the presence of large amounts of polyP. In the meantime, investigation of other cells known to possess dense granules or a V-$H^+$-PPase led to the discovery of similar organelles in other eukaryotic supergroups besides those belonging to the Discoba (trypanosomatids) (14–16), like Amoebozoa (*Dictyostellium discoideum*) (17), Archaeplastida (*Chlamydomonas reinardtii*) (18), Stramenopila-Alveolata-Rizaria [like *Toxoplasma gondii* (19), *Plasmodium* spp. (20), and *Eimeria* spp. (21)], and Opisthokonta {including sea urchins (22), insects (23), and humans [platelets (24) and mast cells (25)]}.

Finally, proteomic analyses of trypanosomatid acidocalcisomes allowed for the identification of numerous transporters, pumps and channels, and some enzymes that made these organelles so peculiar, and defined the mechanisms they use for $Ca^{2+}$ signaling, synthesis, and degradation of polyP, phosphate ($P_i$) release, and accumulation of organic and inorganic cations (26). As expected, it was found that acidocalcisomes and acidocalcisome-like organelles of different species have distinct biochemical compositions, but all have in common the storage of polyP and calcium. The organelle is probably the earliest acidic calcium store that appeared during evolution (27). The study of acidocalcisomes by different groups has shed light on the important roles that these organelles play in cellular processes (28), and these will be the subject of this review.

## PHYLOGENETIC DISTRIBUTION OF ACIDOCALCISOMES

The calcium and polyP storage capacity of acidocalcisomes is also present in the prokaryotic subcellular compartments known as polyP granules. These granules were reported by the end of the 19th century and named metachromatic (1) or volutin (29) granules. These names represented their property of staining red or purple when treated with toluidine blue (metachromatic granules) and the fact that they were first detected in *Spirillum volutans* (volutin granules). Staining with toluidine blue was applied to numerous protists that were then reported to have volutin granules, like yeasts (30, 31) and trypanosomatids (32). The finding that the number of granules within the yeast vacuoles increased when polyP increased led to a change in their name to polyP granules (30). Both bacteria (1, 29) and archaea (33, 34) possess polyP granules.

Early reports suggested that bacterial polyP granules were surrounded by a limiting membrane (35), but a membrane was not detected in other bacteria with current microscopy techniques, and it has been suggested that they resemble membrane-less compartments arising by liquid-liquid phase separation (36, 37). In this regard, polyP has been shown to induce liquid phase separation of proteins (38). However, some bacteria, like *Agrobacterium tumefaciens* (39) and *Rhodospirillum rubrum* (40), have internal membrane-bound vesicles as detected by electron microscopy (EM) of intact bacteria and subcellular fractions. Vesicles were also found in *A. tumefaciens* after they were snap frozen in living cells and then fractured and etched (QFDEEM) (41). Some of these vesicles, which are also detected by staining with dyes that accumulate in acidic compartments (cycloprodigiosin, lysosensor DND 167), are labeled with antibodies against the V-$H^+$-PPase, as determined by immunofluorescence and electron microscopy analyses (39, 40). Subcellular fractionation found co-localization of the V-$H^+$-PPase

activity with polyP as determined by biochemical methods, suggesting that they were acidocalcisome-like organelles (39, 40).

More recent work in *A. tumefaciens* during different phases of growth proposed that acidocalcisomes and polyP granules are different subcellular structures (42). However, some contradictions were reported. Globular dark structures, detected by bright field microscopy, were shown to co-localize with either 4′,6-diamidino-2-phenylindole (DAPI)-fluorescent foci (polyP granules) [Fig. 4 of reference (42)] or the cell pole-located V-$H^+$-PPase-GFP fluorescence [Fig. 5D and E of reference (42)], or with MitoTracker [Fig. 5B of reference (42)], used to detect acidocalcisomes, supporting that they represent the same structures. When the co-localization of DAPI and tagged V-$H^+$-PPase was investigated, the authors found higher expression of the V-$H^+$-PPase tagged with GFP in vesicles during the late stationary phase when DAPI-stainable polyP was rarely present. In the rare cases when both were detected, they were located closely together (42). DAPI cannot stain very short polyP (43, 44), and some acidocalcisomes are known to be rich in this type of polyP [tripolyphosphate (polyP$_3$), polyP$_4$, and polyP$_5$] (11), which could explain the lack of detection of DAPI-positive vesicles in late stationary phase. It would be interesting to investigate whether this could be the reason for the apparent lack of detection of co-localization of both the V-$H^+$-PPase and polyP in some cases.

Interestingly, sulfide-oxidizing bacteria of the genus *Beggiatoa* accumulate polyP and calcium in acidocalcisome-like inclusions surrounded by a lipid layer, although they are not notably acidic (45).

Acidocalcisomes with characteristics similar to those found initially in *Trypanosoma brucei* (4) and *Trypanosoma cruzi* (3) were identified in all trypanosomatids investigated, including digenetic *Leishmania mexicana*, *Leishmania donovani* (46, 47), *Trypanosoma evansi* (48), and *Phytomonas francai* (16), as well as in monogenetic trypanosomatids (49) and *Naegleria gruberi* (41), which also belong to the Discoba "supergroup" of eukaryotes.

Most other major supergroups of eukaryotes have species containing acidocalcisomes. One of the first organisms investigated was *Toxoplasma gondii* (19), which belongs to the Stramenopila-Alveolata-Rizaria supergroup. These acidic $Ca^{2+}$ stores were biochemically characterized (19). A V-$H^+$-PPase was identified (50) and shown to co-localize with a $Ca^{2+}$-ATPase (51). The V-$H^+$-PPase was needed for polyP storage (52). The organelles were isolated (53) and were shown to transport $Ca^{2+}$ and protons (54). Other Alveolata members such *Plasmodium falciparum*, (55), *Plasmodium berghei* (20), and *Eimeria* spp. (21) also possess acidocalcisomes. The presence of polyP in the acidocalcisome-like organelles of ciliates has not been investigated (56, 57).

Within Archaeplastida, which includes algae and plants, the green alga *Chlamydomonas reinhardtii* was known to possess polyP granules within "acidic vacuoles" (58) and a protein that reacted with antibodies against a plant V-$H^+$-PPase and localized to contractile vacuoles, the Golgi complex, and "intermediate-sized vesicles" (59). The measurement of V-$H^+$-PPase activity, which co-localized with a V-$H^+$-ATPase in vacuoles containing polyP determined that these acidic vacuoles or intermediate-sized vesicles were acidocalcisomes (18). Interestingly, although the vacuoles of higher plants also possess a V-$H^+$-PPase and a V-$H^+$-ATPase and several transporters with orthologs present in acidocalcisomes of several species, efforts to find polyP in higher plants have been unsuccessful (60). Other species of *Chlamydomonas* (41) and the green algae *Dunaliella salina* (61) and *Desmodesmus* sp. (62) possess polyP in acidic vacuoles compatible with acidocalcisomes. The red alga *Cyanidioschyzon merolae* was found to possess polyP in vacuoles with morphology similar to that of acidocalcisomes (63). These were isolated using iodixanol gradient centrifugation and shown to contain several pumps (V-$H^+$-ATPase and V-$H^+$-PPase) and transporters (for $Zn^{2+}$ and for $Fe^{2+}$/$Mn^{2+}$) also present in trypanosomatid acidocalcisomes (63).

The supergroup Amoebozoa includes *Dictyostelium discoideum*, which was known to possess "mass-dense granules" rich in phosphorus and calcium (64, 65). Mass-dense granules have polyP, a V-$H^+$-ATPase, and a $Ca^{2+}$-ATPase (Pat1), as occurs with other acidocalcisomes, and a pyrophosphatase with similarities to the V-$H^+$-PPase (17). In

addition, a membrane-bound pyrophosphatase had been described before in *D. discoideum* (66). A protein was detected with heterologous antibodies against the plant V-$H^+$-PPase. However, the pyrophosphatase activity was not completely inhibited by the inhibitors aminomethylenediphosphonate (AMDP) and imidodiphosphate (IDP), and no $PP_i$-driven proton transport was reported (17), suggesting that this was not a typical V-$H^+$-PPase, in agreement with genetic evidence of the absence of the gene in the genome of *D. discoideum* (41). Another amoebozoan, *Entamoeba histolytica*, also possesses electron-dense granules rich in $P_i$, $PP_i$, and cations ($Na^+$, $Mg^{2+}$, $K^+$, $Ca^{2+}$, and $Fe^{2+}$) (67), but the presence of polyP in these acidocalcisome-like organelles has not been investigated.

The supergroup Opisthokonta includes fungi and animals and has species possessing acidocalcisome-like organelles. The yeast vacuole has been proposed as an acidocalcisome-like organelle (68) as it is acidic (it has a V-$H^+$-ATPase but not a V-$H^+$-PPase), rich in polyP and cations, and has several transporters in common with those of trypanosomatid acidocalcisomes. Other fungi, such as *Candida* spp. (69, 70), *Neurospora crassa* (71), and arbuscular mycorrhizal fungi (72, 73), have a vacuolar compartment in the form of small vesicles or tubules rich in polyP and with similarities to acidocalcisomes. Acidocalcisome-like vacuoles were also found in the yolk of insect (23) and chicken (74) eggs and in sea urchin eggs (22). In all these cases, these vacuoles are acidic and rich in polyP and calcium. In humans, it was found that some lysosome-related organelles such as platelet-dense granules (24) and mast cell granules (25) are acidic, rich in $P_i$, $PP_i$, polyP, and calcium and similar to acidocalcisomes.

The evidence reported indicates that acidocalcisomes and acidocalcisome-like vacuoles are widely distributed and reveals the importance of polyP during evolution.

## STRUCTURAL CHARACTERISTICS OF ACIDOCALCISOMES

Acidocalcisomes were first identified in trypanosomes by their staining with acridine orange that was prevented by the V-$H^+$-ATPase inhibitor bafilomycin $A_1$ or the $K^+/H^+$ exchanger nigericin (3, 4). Later work (5, 14) demonstrated that the acidic vacuoles of *Trypanosoma cruzi* and *T. brucei* corresponded to the electron-dense vacuoles detected by electron microscopy (Fig. 1A through C) that, when analyzed by X-ray microanalyses, were found to be rich in cations ($Ca^{2+}$, $Mg^{2+}$, $K^+$, $Zn^{2+}$, and $Fe^{2+}$), oxygen, and phosphorus (7) (Fig. 1D). Similar electron-dense compartments had been described before in *Trypanosoma cyclops* (8) and *Leishmania major* (6).

The use of electron microscopy led to the identification of the acidocalcisomes by conventional electron microscopy as "empty" vacuoles sometimes surrounded by a peripheral dense region or showing interior electron-dense granules (75) (Fig. 1A and B). The appearance of empty vacuoles is due to the procedure applied for electron microscopy preparation that depletes their content. By cryo-electron microscopy, however, acidocalcisomes appear as electron-dense spheres (5). Depositing these protists on a grid, letting them dry, and observing them by EM also allow their detection in intact form as electron dense spheres (5) (Fig. 1C). Their size has been measured in different trypanosomatids (16, 49, 75) and varies from ~50 to ~250 nm in diameter reaching values of ~600 nm in diameter in *Leishmania* spp. (76). They occupy ~2% of the total cell volume (49). Some acidocalcisomes are elongated, like in *Phytomonas* spp. (16, 49), or pleomorphic, such as those in *Leishmania mexicana amazonensis*, grown in a deficient culture medium (76). There is no apparent specific distribution in the cells and their numbers are variable. In *T. brucei* bloodstream forms, there are about 40 acidocalcisomes per cell at the start of the cell cycle and their numbers increase to ~56 prior to cell division (77).

The ultrastructural analysis of acidocalcisomes of the alga *Chlamydomonas reinhardtii* and other protists was undertaken using living cells snap-frozen at liquid helium temperatures, subjected to freeze-fracture, deep etching, and platinum rotary-replication, and observed by transmission electron microscopy (41). This technique allowed the identification of a large population of intramembranous particles in the P-fracture concave face of the membrane (contiguous to the cytoplasm) and attributed them to

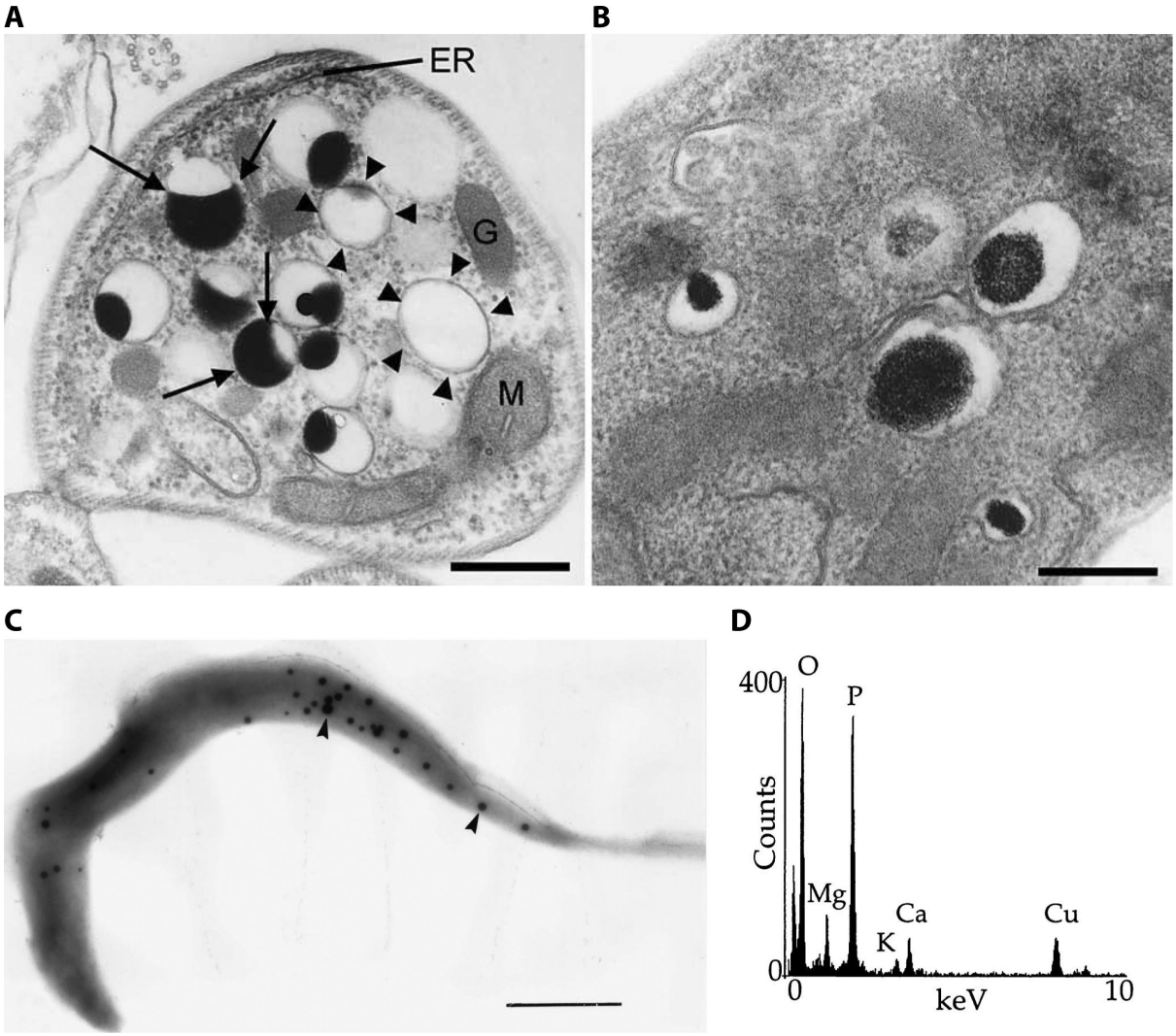


**FIG 1** Ultrastructure of acidocalcisomes. (A and B) Thin sections of *Leishmania amazonensis* (A) and *Herpetomonas anglusteri* (B) submitted to conventional transmission electron microscopy. Acidocalcisomes are partially filled with electron-dense material ("granule") (arrows) or appear empty or with an electron-dense periphery (arrowheads). Bars are 280 nm (A) or 130 nm (B). (C) transmission electron microscopy of whole bloodstream trypomastigotes of *T. brucei*. The arrowheads indicate acidocalcisomes. Bar, 2 µm. (D) X-ray microanalysis spectrum of dense organelles in whole bloodstream trypomastigotes. (Panels A and B were reproduced from reference 49 with permission from Elsevier; panels C and D were reproduced from reference 14 with permission from Taylor & Francis Informa UK Ltd.) ER, endoplasmic reticulum; G, glycosome; M, mitochondrion.

intramembrane proteins (Fig. 2). The convex E-fracture face (contiguous to the lumen) was smooth or rugose, depending on its exposure to etching. An interior granule, attributed to polyP in a gel configuration, was detected in stationary phase cells or in those submitted to nitrogen (N)-starvation (41) (Fig. 2). The authors also investigated the distribution of V-$H^+$-PPase-encoding genes in eukaryotes, finding three clades and that clade-1 proteins ($K^+$-stimulated) are acidocalcisome associated (41).

Acidocalcisomes have also been analyzed by cryofixation of cells and scanning transmission electron microscopy tomography combined with elemental mapping using a high-performance setup of X-ray detectors (78). The work reported that the elemental distribution was not homogenous but rather organized in nanodomains within the organelles with cationic elements displaying a self-excluding pattern (78).

In summary, the structural characteristics of acidocalcisomes are quite peculiar and different from any other organelle.

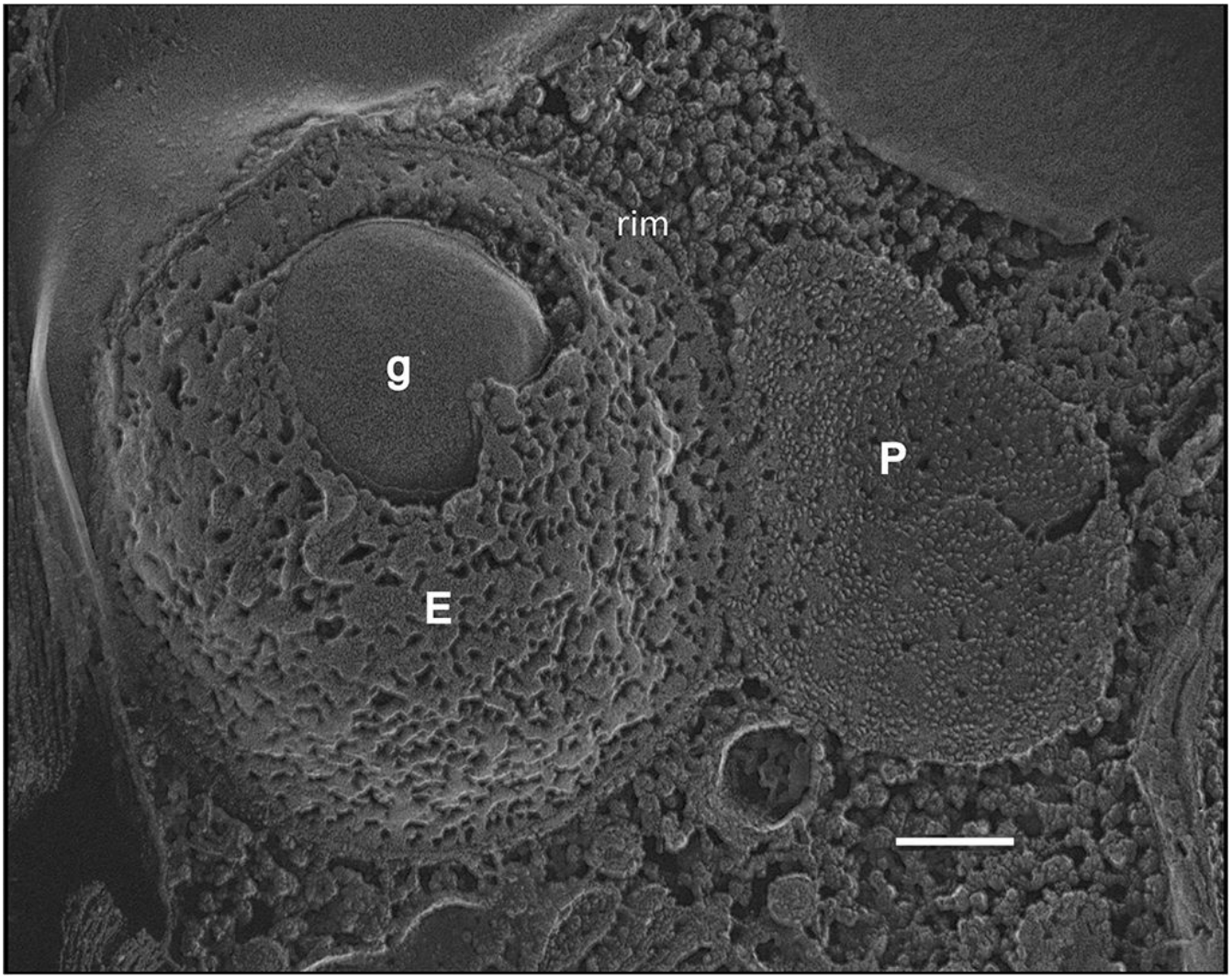


**FIG 2** Cross-fractured acidocalcisomes. Two acidocalcisomes contiguous to one another from log phase *C. reinhardtii* N-starved for 24 h. The fracture plane exposed the P-face of the right organelle and the E-face of the left organelle. A large population of intramembranous particles is in the P-face of the membrane. The convex E-fracture face is rugose. "Rim" denotes the outer edge. Bar, 100 nm. P, P-fracture; E, E-fracture; g, polyphosphate granule. (Reproduced from reference 41 with permission from Elsevier.)

## CHEMICAL COMPOSITION OF ACIDOCALCISOMES

The acidocalcisome matrix is rich in $P_i$, $PP_i$, and polyP complexed with organic and inorganic cations and has a few enzymes. The phosphorus compounds have been identified by $^{31}P$ NMR of isolated organelles from different trypanosomatids (11), and apicomplexan (79) parasites, or by labeling some of these cells with $^{32}P_i$ (13). Very short-chain polyP (polyP$_3$, PolyP$_4$, and polyP$_5$) was detected by $^{31}P$ NMR in trypanosomatids (11). The results suggested that the average chain length of polyP is of ~3.2 phosphates. Longer-chain polyP was more difficult to identify. However, using polyacrylamide gel electrophoresis analyses of isolated acidocalcisomes from *T. brucei* it was possible to also identify the presence of short-chain (<300 mer) and long-chain (>300–1,000 mer) polyP (80).

The presence of organic cations, mainly basic amino acids like arginine, lysine, and ornithine, was demonstrated in acidocalcisomes from *T. cruzi* epimastigotes, where they represent about 90% of the total amino acid pool (81). Although the presence of polyamines has not been reported in acidocalcisomes of trypanosomatids, they

possess polyamine transporters (26) as the acidocalcisome-like vacuoles of *Saccharomyces cerevisiae*, which are rich in these cationic compounds (82, 83).

Inorganic cations ($Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, $K^{+}$, and $Zn^{2+}$) are also abundant in acidocalcisomes of most species investigated by X-ray microanalysis methods. *Chlamydomonas reinhardtii* acidocalcisomes are also rich in copper (84), manganese (85), and iron (86), while iron has been found in acidocalcisomes of *Phytomonas* spp., *T. cruzi*, and other trypanosomatids (16, 49, 75, 76). Some metals, like manganese, iron, and copper, could be present in smaller amounts in some organisms, below the detection limit of X-ray microanalysis, as these cells possess transporters for them. Liquid chromatography (LC) studies of the acidocalcisome-like vacuoles of yeast have demonstrated that most of the iron, zinc, and manganese (and some copper) are coordinated to polyP chains (87). The results indicated that these metals are present in vacuoles in low-molecular-mass forms that migrate according to masses ranging from 500 to 10,000 Da. The observed LC peaks comigrated with phosphorus, supporting the presence of metal-polyP complexes. Treatment with polyphosphatase disrupted these complexes. In some cases, the metals comigrated with each other, suggesting that multiple metals either bind to the same chain or to different chains of about the same length. The length distribution of polyP chains in the samples was of chains of 6–20 units long, suggesting that these lengths were either especially stable or produced at especially high rates. The authors suggested that these preferences must reflect the steady-state distribution resulting from opposing polyP synthesis and degradation processes or that metals may also preferentially bind to and/or stabilize chains of specific lengths (87).

Isolated acidocalcisomes from different trypanosomatids (*T. brucei*, *T. cruzi*, and *Leishmania major*) were also studied by magic-angle spinning $^{31}P$ NMR spectroscopy and resulted in the detection of condensed phosphates with the dominant presence of $polyP_3$ and low abundance of long-chain polyP (12).

Lipid analysis of highly purified acidocalcisomes from *T. cruzi* (88) showed very low amounts of 3β-hydroxysterols. Alkylacyl phosphatidylinositol (16:0/18:2), diacyl phosphatidylinositol (18:0/18:2), diacyl phosphatidylcholine (16:0/18:2; 16:1/18:2; 16:2/18:2; 18:1/18:2, and 18:2/18:2), and diacyl phosphatidylethanolamine (16:0/18:2 and 16:1/18:2) were identified by electrospray ionization-mass spectrometry (88). A glycoinositolphospholipid (GIPL) was also detected with a structure apparently different from the GIPL found in microsomal fractions (88).

Proteomic analysis of *T. brucei* acidocalcisomes (26) and N-terminal or C-terminal tagging of proteins by the TrypTag.org project (89, 90) were able to identify more than 30 proteins. The acidocalcisome localization was validated for some of them by immunofluorescence co-localization with the V-$H^{+}$-PPase (Table 1; Fig. 3). Figure 4 shows the scheme of a *T. brucei* acidocalcisome.

*T. cruzi* has orthologs to most acidocalcisome proteins of *T. brucei*, and their localization was in part determined by proteomic analysis (103). Co-localization studies validated some of them. A few proteins with no acidocalcisome orthologs in *T. brucei* have also been found in acidocalcisomes of *T. cruzi* (Table 2).

Proteomic analysis of *C. merolae* acidocalcisomes, then called "polyP vacuoles," identified several orthologs to proteins present in acidocalcisomes of *T. brucei*, like vacuolar $H^{+}$-ATPase subunits, vacuolar $H^{+}$-PPase (CMO102C), endopolyphosphatase (CMG087C), acid phosphatase (CM7279C), zinc transporter (CMF058C), vacuolar iron transporter (CMT466C), and vacuolar transporter chaperone 1 (CMP062C) (63). Antibodies against an M13 family metallopeptidase (CMP249C) or against C-terminal HA-tagged *o*-methyltransferase (CMT369C), ABC transporter (CMS401C), and prenylated Rab receptor (CMJ260C) co-localized with the vacuolar $H^{+}$-ATPase to these vacuoles, as detected by immunofluorescence analyses (63).

Early work on *C. reinhardtii* acidocalcisomes reported the isolation of the then called "polyphosphate bodies" with detection of $PP_i$ and polyP using $^{31}P$ NMR, and phosphorus, magnesium, and calcium, using X-ray microanalysis (58). A PPase activity was also measured in microsomal fractions, and antibodies against the plant V-$H^{+}$-PPase localized

**TABLE 1** Acidocalcisome localization of *T. brucei* proteins, validated by marker co-localization

| Gene | Name | TMD[d] | MW[e] | References |
|---|---|---|---|---|
| Tb927.11.11160 | Phosphate transporter 91 (fragment) (PHO91) | 8 | 81.451 | Huang et al. (26) |
| | | | | Billington et al. (89)[a] |
| Tb927.9.10340 | Polyamine transporter 1 | 11 | 54.065 | Huang et al. (26) |
| Tb927.3.800 | Vacuolar iron transporter | 3 | 30.573 | Huang et al. (26) |
| | | | | Billington et al. (89)[a] |
| Tb927.4.4960 | $Zn^{2+}$ transporter (ZnT1) | 5 | 50.697 | Huang et al. (26) |
| Tb927.8.7460 | $Zn^{2+}$ transporter (ZnT2) | 5 | 50.823 | Billington et al. (89)[a] |
| Tb927.9.5490 | Cation transporter ($Mg^{2+}$) | 1 | 75.937 | Billington et al. (89)[a] |
| Tb927.5.1260 | Sulfate transporter | 11 | 62.563 | Billington et al. (89)[a] |
| Tb927.11.16630 | Major facilitator superfamily MFS,[b] nodulin-like | 14 | 74.876 | Billington et al. (89)[a] |
| Tb927.11.12270 | MFS, drug resistance protein | 10 | 53.057 | Billington et al. (89)[a] |
| Tb927.10.12400 | Multidrug and Toxic extrusion protein | 11 | 51.410 | Billington et al. (89)[a] |
| Tb927.7.3900 | Vacuolar transporter chaperone 1 | 3 | 19.768 | Fang et al. (91) |
| Tb927.11.12220 | Vacuolar transporter chaperone 4 | 2 | 91.362 | Lander et al. (92) |
| | | | | Ulrich et al. (93) |
| | | | | Huang et al. (26) |
| | | | | Billington et al. (89)[a] |
| Tb927.11.1260 | Cu-ATPase (ATP7) | 8 | 102.336 | Isah et al. (94); Paul et al. (95) |
| | | | | Billington et al. (89)[a] |
| Tb927.8.1160 | $Ca^{2+}$-ATPase (PMC1) | 10 | 121.242 | Luo et al. (96) |
| Tb927.4.4380 | Vacuolar $H^+$-PPase 1 | 16 (SP) | 85.936 | Rodrigues et al. (14) |
| | | | | Billington et al. (89)[a] |
| Tb927.8.7980 | Vacuolar $H^+$-PPase 2 | 16 | 86.004 | Rodrigues et al. (14) |
| | | | | Billington et al. (89)[a] |
| Tb927.5.1300 | Vacuolar $H^+$-ATPase (subunit *a*) | 6 | 89.631 | Vercesi et al. (4) |
| | | | | Huang et al. (26) |
| Tb927.5.550 | Vacuolar $H^+$-ATPase (subunit D) | – | 42.849 | Vercesi et al.(4) |
| | | | | Huang et al. (26) |
| Tb927.8.2310 | Vacuolar $H^+$-ATPase (subunit G) | – | 12.749 | Vercesi et al. (4) |
| | | | | Billington et al. (89)[a] |
| Tb927.4.1080 | Vacuolar $H^+$-ATPase (subunit A) | – | 67.750 | Vercesi et al. (4) |
| | | | | Billington et al. (89)[a] |
| Tb927.11.11690 | Vacuolar $H^+$-ATPase (subunit B) | – | 55.620 | Vercesi et al. (4) |
| | | | | Billington et al. (89)[a] |
| Tb927.8.2770 | Inositol trisphosphate receptor | 5 | 342.483 | Huang et al. (97) |
| | | | | Billington et al. (89)[a] |
| Tb927.11.12490 | Potassium channel (IRK) | 3 | 61.904 | Steinmann et al. (98) |
| | | | | Billington et al. (89)[a] |
| Tb927.11.7080 | Vacuolar soluble pyrophosphatase | – | 47.297 | Lemercier et al. (99) |
| | | | | Huang et al. (26) |
| Tb927.10.7020 | Acid phosphatase | – | 49.918 | Huang et al. (26) |
| Tb927.6.4630 | Kinetoplastid-specific phosphoprotein phosphatase (Ppn2) | 1 | 39.849 | Huang et al. (26) |
| | | | | Billington et al. (89)[a] |
| Tb927.11.10650 | AP-3[c] β3 subunit | – | 100.360 | Huang et al. (100) |
| Tb927.5.3610 | AP-3 δ subunit | 1 | 125.404 | Huang et al. (100) |
| Tb927.10.10800 | Palmitoyl acyl transferase 2 | 3 | 49.905 | Huang et al. (26); |
| | | | | Billington et al. (89)[a] |
| | | | | Emmer et al. (101) |
| Tb927.10.6180 | FLA1-like protein | 1 | 54.878 | Huang et al. (26) |
| | | | | Sun et al. (102) |

(*Continued on next page*)

**TABLE 1** Acidocalcisome localization of *T. brucei* proteins, validated by marker co-localization (*Continued*)

| Gene | Name | TMD[d] | MW[e] | References |
|---|---|---|---|---|
| | | | | Billington et al. (89)[a] |
| Tb927.10.9560 | Oxidoreductase | – | 36.100 | Billington et al. (89)[a] |
| Tb927.11.1890 | Serine threonine kinase-associated teceptor | – | 36.025 | Billington et al. (89)[a] |
| Tb927.4.860 | Hypothetical protein | – | 33.145 | Billington et al. (89)[a] |
| Tb927.4.5335 | Hypothetical protein | – | 10.705 | Billington et al. (89)[a] |
| Tb927.8.6990 | Hypothetical protein | – | 10.759 | Billington et al. (89)[a] |
| Tb927.9.12070 | Hypothetical protein | – | 109.870 | Billington et al. (89)[a] |
| Tb927.10.11810 | Hypothetical protein | – | 69.988 | Billington et al. (89)[a] |
| Tb927.11.8250 | Hypothetical protein | 1 | 19.345 | Billington et al. (89)[a] |

[a]Tentative localization based on immunofluorescence analysis of C-terminal or N-terminal tagged proteins (TrypTag project).
[b]MFS, major facilitator superfamily.
[c]AP-3, adaptor protein 3.
[d]TMD, transmembrane domain.
[e]MW, molecular weight.

a protein to intermediate-sized vesicles, plasma membranes, and contractile vacuoles (59). Later work that included $^{45}Ca$ release experiments from acidic compartments, organelle isolation using iodixanol gradient centrifugation, X-ray microanalysis of isolated or *in situ* organelles, $PP_i$-driven proton transport measurements, and immunofluorescent analysis revealed that these intermediate-sized vesicles were acidocalcisomes and that they possess a V-$H^+$-PPase and a V-$H^+$-ATPase (18). In a recent work, acidocalcisomes were isolated by a modified iodixanol gradient centrifugation method, and proteomic analysis of the fractions obtained was done (86). Two types of vacuoles were obtained and named lysosome-related organelles from stationary phase (stat-LROs) and iron-loaded lysosome related organelles (Fe-LROs) or ACs. The difference between these fractions is that the Fe-LROs were obtained from cells incubated at high iron concentration (>200 µM) for 1 day after partially starving them of iron for 5 days (<50 µM), while the stat-LROs were cultured for 6 days in a regular culture medium (86). Surprisingly, the authors considered them as different organelles. However, the stat-LRO appeared as acidocalcisomes surrounded by portions of cytoplasm [Fig. 1E of reference (86)], contained deformed mitochondria and cellular debris [Fig. S5E and D of reference (86)], and had autophagy markers (ATG8), suggesting that they are autophagosomes. The localization of four proteins [inorganic pyrophosphatase (IPPase, Cre10.g424100.t1.2), copper transporter (CRT1, Cre13.g570600.t1.2), V-$H^+$-ATPase (vATPase subunit C/D,

**TABLE 2** Validated acidocalcisome proteins present in *T. Cruzi*[a]

| Gene | Name | TMD[b] | MW[c] | References |
|---|---|---|---|---|
| TcCLB.511439.50 | $Zn^{2+}$ transporter 1 | 5 | 49.901 | Ferella et al. (103) |
| TcCLB.511127.100 | Vacuolar transporter chaperone 4 | 2 | 91.558 | Ulrich et al. (93) |
| TcCLB.506401.170 | $Ca^{2+}$-ATPase (PMC1) | 8 | 121.912 | Lu et al. (104) |
| TcCLB.510773.20 | Vacuolar $H^+$-PPase 1 | 16 | 85.338 | Scott et al. (9) |
| TcCLB.508257.40 | Aquaporin 1 | 6 | 24.711 | Montalvetti et al. (105)<br>Rohloff et al. (106) |
| TcCLB.509461.90 | Inositol trisphosphate receptor | 5 | 337.362 | Lander et al. (107)<br>Chiurillo et al. (108) |
| TcCLB.503613.60 | Vacuolar soluble pyrophosphatase | – | 47.854 | Gallizi et al. (109) |
| TcCLB.506311.20 | Palmitoyl acyl transferase 2 | 4 | 51.182 | Batista et al. (110) |
| TcCLB.506247.220 | Histidine ammonia-lyase | – | 58.055 | Mantilla et al. (111) |

[a]Aquaporin and histidine-ammonia lyase are not present in *T. brucei* acidocalcisomes.
[b]Transmembrane domain (TMD).
[c]Molecular weight (MW).

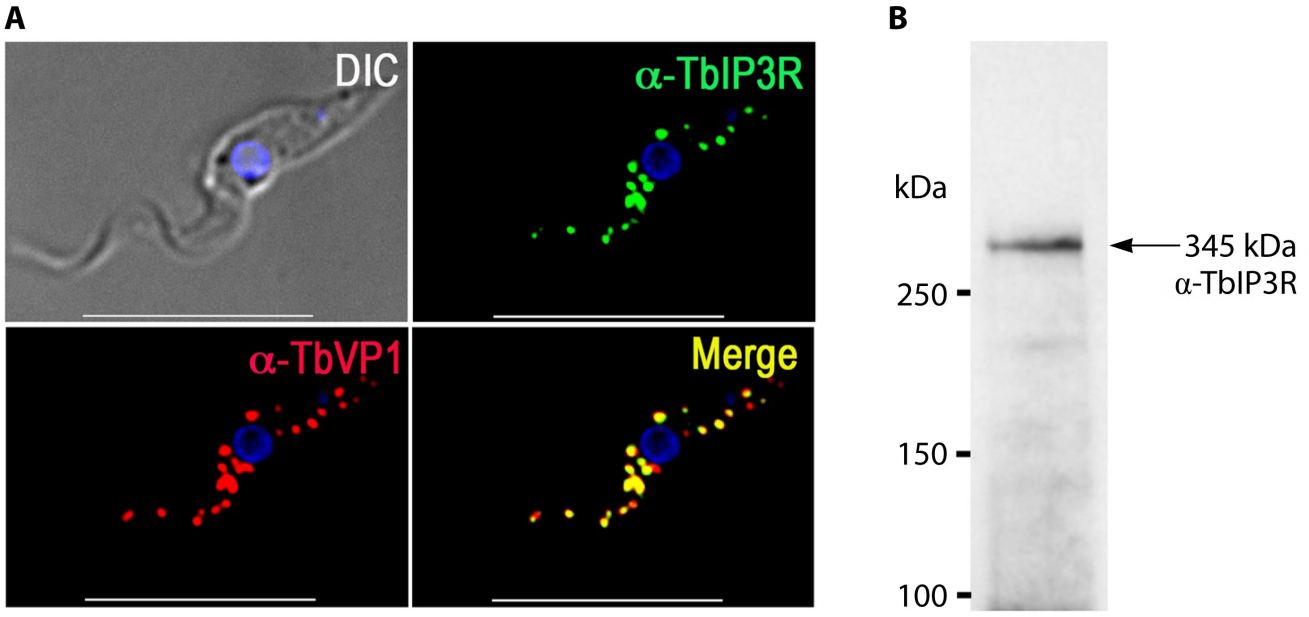


**FIG 3** Immunofluorescence analysis of *T. brucei*. (A) TbIP$_3$R co-localizes with the V-H$^+$-PPase in acidocalcisomes of procyclic (PCF) trypomastigotes (Pearson's correlation coefficient of 0.8399). Yellow in merge images indicates co-localization. Scale bars: 10 µm. (B) Western blot analysis of TbIP3R expressed in PCF trypanosomes using polyclonal anti-TbIP$_3$R antibody. Lysate containing 30 mg of protein from PCF trypanosomes was subjected to SDS/PAGE on 4%–15% polyacrylamide gel and transferred to a nitrocellulose membrane. Molecular weight markers on the left and arrow shows the band corresponding to TbIP$_3$R. (Reproduced from reference 26.) TbIP3R, *T. brucei* IP$_3$R.

Cre03.g176250.t1.2), and isocitrate lyase (CL1, Cre06.g282800.t1.2)] to acidocalcisomes was validated by their co-localization with DAPI-stained polyP and LysoTracker (86). A number of potential acidocalcisome proteins with similarity to those found in *T. brucei* acidocalcisomes (metal transporters, phosphate transporters, and $Ca^{2+}$-ATPases) were found in these fractions (86) and await validation.

In addition to the above studies, several publications have reported the finding of proteins homologous to those in acidocalcisomes of *T. brucei* (Table 1) and postulated their presence in acidocalcisome-like organelles. The V-H$^+$-PPase was found in acidocalcisomes of *Leishmania donovani* (47), *Plasmodium falciparum* (112), *P. berghei* (20), *T. gondii* (50), *Agrobacterium tumefaciens* (39), *L. mexicana amazonensis* (76), *Rhodospirillum rubrum* (40), *Phytomonas francai* (16), and *Eimeria tenella* (21). A $Ca^{2+}$-ATPase (51) and a zinc transporter (113) were found in acidocalcisomes of *T. gondii*. A Cu-ATPase (ATP7) was found near acidocalcisomes of *P. berghei* or co-localized with the V-H$^+$-PPase to acidocalcisomes and the plant-like vacuole (PLVAC) of *T. gondii* (114).

We can conclude that the chemical composition of acidocalcisomes is well adapted to their storage function of phosphorus and cations. They have a reduced number of matrix proteins, as compared to other organelles, and several transporters involved in their store function.

## ACIDOCALCISOME PUMPS, TRANSPORTERS, ENZYMES, AND CHANNELS

### The proton pumps

Acidocalcisomes of some protists (some trypanosomatids, apicomplexan parasites, and algae) possess two electrogenic proton pumps, a V-H$^+$-PPase and a V-H$^+$-ATPase, while other acidocalcisomes possess either a V-H$^+$-PPase or a V-H$^+$-ATPase.

The V-H$^+$-ATPase is a multisubunit complex of about 14 subunits and 2 sectors, the membrane ($V_0$) and the catalytic ($V_1$) sectors. It was originally identified in acidocalcisomes of *T. brucei* (4) and *T. cruzi* (3) by the proton pumping and acridine orange staining inhibition by bafilomycin $A_1$ in permeabilized cells. It was also functionally identified in

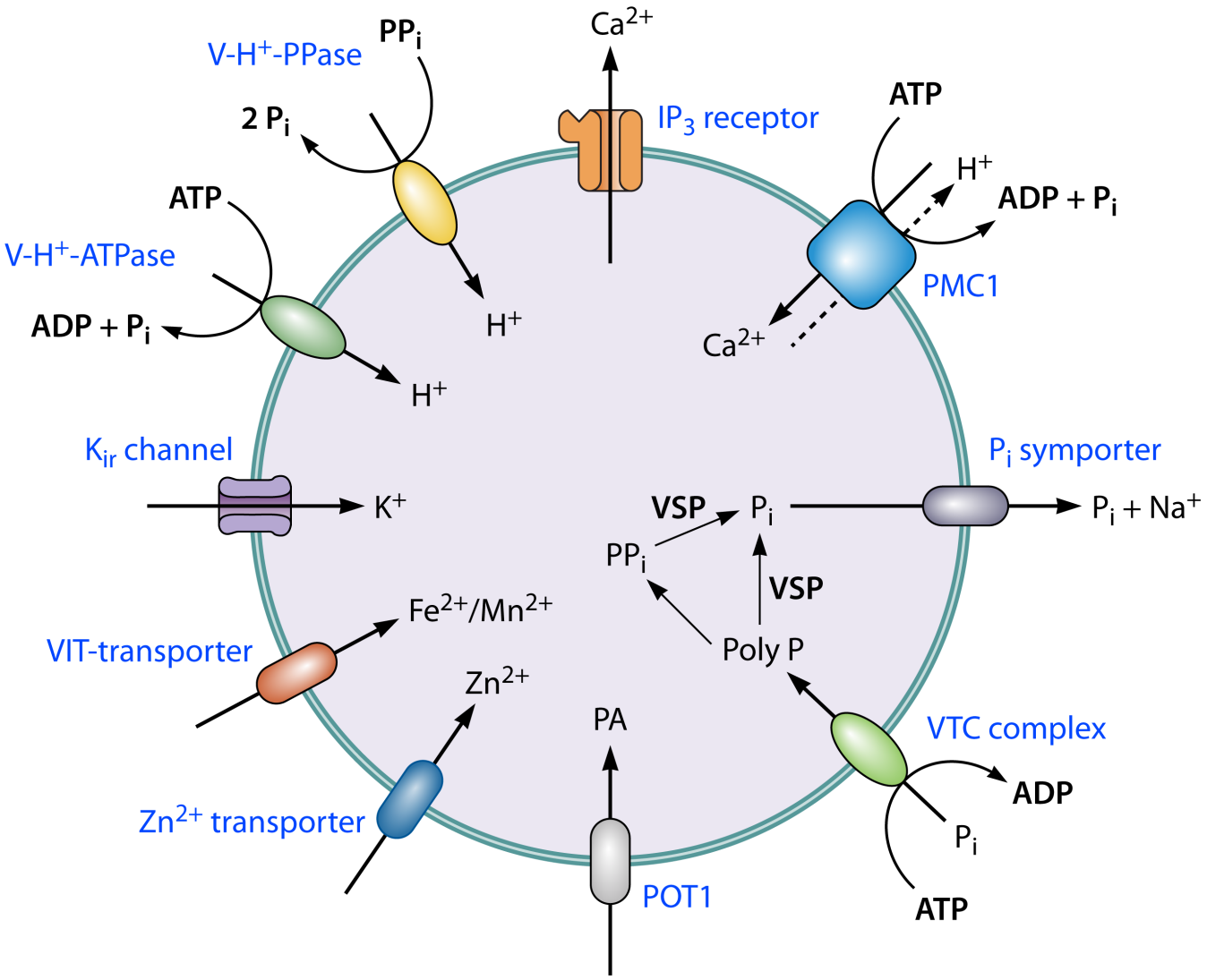


**FIG 4** Scheme of an acidocalcisome of *T. brucei* with validated transporters and channels. Uptake of $Ca^{2+}$ is through a $Ca^{2+}$-ATPase, and $Ca^{2+}$ release is by the $IP_3$ receptor. $H^+$ is pumped in by either the V-$H^+$-PPase or the V-$H^+$-ATPase. A vacuolar iron transporter (VIT1) can transport either $Mn^{2+}$ or $Fe^{2+}$, and two $Zn^{2+}$ transporters (ZnT1 and ZnT2, not shown) transport $Zn^{2+}$. There is a PA transporter and $K^+$ channel. A VTC complex synthesizes polyP using ATP and translocates it into the organelle. A $Na^+$/Pi symporter (Pho91) releases $Na^+$ and $P_i$. Within acidocalcisomes, there is a VSP with pyrophosphatase and exopolyphosphatase activity. Several adaptor protein 3 complex subunits localize to the acidocalcisome (not shown). PA, polyamine; VSP, vacuolar soluble pyrophosphatase; VTC, vacuolar transporter chaperone.

acidocalcisomes of *T. evansi* (48), *T. gondii* (19, 115, 116), *C. reinhardtii* (18), *D. discoideum* (17), and human platelets (24). Immunofluorescence and/or immunoelectron microscopy studies confirmed the acidocalcisome localization of several subunits in *T. brucei* (Table 1), *T. cruzi* (117), *T. gondii* (118), *C. reinhardtii* (18), *C. merolae* (63), and *D. discoideum* (17). The complex has other subcellular localizations such as the Golgi complex, the plasma membrane, and the endocytic pathway of *T. brucei* (26), the plasma membrane of *T. cruzi* (117), the plasma membrane and the PLVAC of *T. gondii* (116), the plasma membrane and the digestive vacuole of malaria parasites (119–122), and the contractile vacuoles of *C. reinhardtii* (18) and *D. discoideum* (17). Downregulation of the expression of some V-$H^+$-ATPase subunits has therefore multiple phenotypic changes that cannot be attributed solely to the disruption of acidocalcisome functions.

The V-$H^+$-PPase is a single subunit $H^+$ pump that uses $PP_i$ to establish a $H^+$ gradient and it was first identified in acidocalcisomes of *T. cruzi* (9). Previously, this pump had been described only in bacteria, archaea, and plants (123). Structural studies of *Vigna radiata*

V-$H^+$-PPase showed that it is present as a homodimer with 16 transmembrane domains (124). The pump is also localized to the Golgi complex and to the plasma membrane of *T. cruzi* (3, 125), and could be heterologously expressed in yeast, which lacks the pump (126). Its activity is stimulated by $K^+$, and inhibited by $Na^+$ and pyrophosphate analogs, like IDP and AMDP, as occurs with the plant pump (9). As the pump is very abundant in acidocalcisomes, it has been used as a marker for subcellular fractionation of the organelle (10, 26). *T. brucei* possess two genes expressing very similar proteins (TbVP1 and TbVP2) (127), both localized to acidocalcisomes (14, 89) (Table 1).

Two genes are also expressed in malaria parasites, but the encoded proteins are different. One corresponds to the $K^+$-stimulated type (VP1, type 1) while the other has homology to the plant $K^+$-insensitive type (VP2, type II) (128). The immunofluorescence localization of *P. falciparum* VP1 in "intracellular bright spots" (112) and "punctate intracellular inclusions" (128), and the localization of *P. berghei* VP1 in "intracellular vacuoles" (20) using antibodies against the plant pump, provided indirect evidence of a potential acidocalcisome localization. The pump was also localized to the plasma membrane (112, 128) and to the digestive vacuole (120) of *P. falciparum*. Two genes are also present in the scuticociliate *Philasterides decentrarchi*, which have homology to genes encoding $K^+$-sensitive (AVP1) and $K^+$-insensitive (AVP2) plant enzymes, the first protein product localized to acidocalcisome-like organelles and the second localized to the alveolar sacs (57).

The evidence for the acidocalcisome localization of the V-$H^+$-PPase in *T. gondii* is stronger. Immunoelectron microscopy revealed that antibodies against the enzyme co-localized with antibodies against a $Ca^{2+}$-ATPase to their acidocalcisomes (51). Acidocalcisome fractions were also shown to contain the AMDP-sensitive PPase activity and $PP_i$-driven acridine orange uptake (54). Interestingly, the V-$H^+$-PPase also localizes to the PLVAC of *T. gondii* (129), where the V-$H^+$-ATPase is also present (118).

There is also strong evidence for the presence of a V-$H^+$-PPase in acidocalcisomes and contractile vacuoles of *C. reinhardtii*, where it co-localizes with the V-$H^+$-ATPase (18).

It is interesting to note that the presence of a V-$H^+$-ATPase and a V-$H^+$-PPase in the same compartment occurs in several organelles such as the plant vacuole (10, 130), the contractile vacuoles of *C. reinardtii* (18), and *T. cruzi* (106), the acidocalcisomes of some trypanosomatids (127), the digestive vacuole of malaria parasites (120), and the PLVAC of *T. gondii* (129).

The function of these proton pumps in acidocalcisomes is to acidify the organelles and allow secondary transporters to export $H^+$ in exchange of different cations, or to export $H^+$ together with $P_i$ by the $P_i$ symporter (Pho91) present in some species (131). The low pH also contributes to maintain the solubility of polyP. In the case of the V-$H^+$-PPases, they also contribute to maintain low levels of cytosolic $PP_i$ and then facilitate anabolic reactions, which produce $PP_i$ (132). A third function could be the generation of a membrane potential that could be used to reverse the V-$H^+$-ATPase and generate ATP or reverse the V-$H^+$-PPase to generate $PP_i$, reactions that could occur *in vitro* (133, 134) but which occurrence *in vivo* has not been established. In this regard, it has been shown that $PP_i$ can generate a membrane potential in acidocalcisomes of *T. brucei* and *T. cruzi* that can be reversed by FCCP or CCCP and prevented by AMDP (10, 14).

## The P-type ATPases

Two P-type ATPases have been reported in acidocalcisomes of several species, a $Ca^{2+}$ATPase and a $Cu^{2+}$-ATPase.

A $Ca^{2+}$-ATPase activity was initially detected in acidocalcisomes of permeabilized *T. brucei* procyclic forms by measuring ATP-driven $Ca^{2+}$ transport inhibited by vanadate (4). The stimulation of $H^+$ uptake (acridine orange uptake) by the $Ca^{2+}$ chelator EGTA and its release by $Ca^{2+}$ suggested their accumulation in the same compartment. In addition, $H^+$ uptake was stimulated by vanadate and vanadate inhibited the release of $H^+$ by $Ca^{2+}$, confirming that the $Ca^{2+}$-ATPase takes up $Ca^{2+}$ in exchange for $H^+$ (4). Further work identified a gene encoding an acidocalcisome-located pump that was named PMC1

(for plasma membrane calcium ATPase) because it was closely related to the family of plasma membrane calcium ATPases (PMCA) (96). Its functional role was demonstrated by its ability to complement yeast deficient in the vacuolar $Ca^{2+}$-ATPase *PMC1* (96). The *T. brucei* pump apparently lacks the C-terminal calmodulin (CaM)-binding domain present in other PMCA pumps (96). Similar $Ca^{2+}$-ATPases were found in acidocalcisomes of *T. cruzi* (Tca1) (104), *T. gondii* (TgA1) (51), and *D. discoideum* (PAT1) (17, 135), and in vacuoles of *S. cerevisiae* (PMC1) (136) and *E. histolytica* (137). Both *T. cruzi* (104) and *T. gondii* (52) $Ca^{2+}$-ATPases were able to complement yeasts deficient in *PMC1,* providing functional demonstration of their activity. All of them, including PAT1 (135), lack the C-terminal CaM-binding domain although it cannot be ruled out that they could have a non-canonical CaM-binding domain as it has been reported in the *T. equiperdum* plasma membrane $Ca^{2+}$-ATPase (138). In this regard, the *T. cruzi* PMC1 from plasma membrane vesicles was shown to be stimulated by endogenous or mammalian calmodulin (139).

A Cu-ATPase (ATP7) was first detected in the plasma and organellar membranes of *T. brucei* (94). The intracellular distribution agrees with its acidocalcisome localization by the TrypTag project (89) (Table 1). The N-terminal region of the protein was shown to bind copper *in vitro* and within *E. coli* cells (94). *Leishmania major* ortholog (LmATP7, LmjF33.2090) also localized to vesicles compatible with acidocalcisomes, in addition to the plasma membrane (95). It has been proposed that vesicles containing copper fuse to the plasma membrane to export the metal (140). The essentiality of the CuATPase of *T. brucei* has not been tested, while complete knockout (KO) of *L. major* CuATPase was not possible, suggesting its essentiality (95). Interestingly, copper accumulates in acidocalcisomes of *C. reinhardtii* during zinc limitation, but the potential role of a CuATPase has not been investigated (141). *P. berghei* and *T. gondii* CuATPases are also detected in intracellular storage vesicles, which could be acidocalcisomes (114).

## PolyP synthesis and degradation

Synthesis and translocation of polyP into the lumen of acidocalcisomes is catalyzed by the vacuolar transporter chaperone (VTC) complex (142). The VTC complex is present in fungi, trypanosomatids, apicomplexans, and algae but is absent in animals. The VTC complex of *S. cervisiae* has five subunits (Vtc1–Vtc5), of which Vtc4 is the catalytic subunit and forms subcomplexes with Vtc1 and Vtc2 or Vtc3 (Vtc1/Vtc2/Vtc4 or Vtc1/Vtc3/Vtc4), which localize to the vacuole membrane (143). Vtc5 does not form part of these complexes but stimulates their activity. Structural studies of these subcomplexes in yeasts have shown that Vtc1 has three transmembrane domains, while Vtc2, Vtc3, and Vtc4 contain additional SPX (SYG1/Pho81/XPR1) and TTM (triphosphate tunnel metalloenzyme) domains (142, 144, 145). The TTM domain of Vtc4 synthesizes polyP by transferring the γ-phosphate of cytosolic ATP onto the growing polyP chain (142). The SPX domains are receptors for cytosolic inositol pyrophosphates (PP-IPs), which stimulate polyP synthesis (143). PP-IPs bind to the Vtc2 SPX domain, preventing its interaction with the SPX domain of Vtc4 and stimulating its activity (146). The VTC complex has therefore functions of polyP polymerase, polyP translocase, and PP-IPs receptor (144) (Fig. 5).

Trypanosomatid acidocalcisomes possess orthologs to Vtc1 and Vtc4, which have been studied in *T. brucei* (91–93), *T. cruzi* (93), and *Leishmania* spp. (147, 148). Vtc1 is the smaller subunit of the VTC complex, and the *T. brucei* protein (TbVtc1) also has three transmembrane domains. The GFP-tagged protein was shown to localize to the acidocalcisomes and endoplasmic reticulum (ER) of *T. brucei* procyclic forms by immunofluorescence assays (IFAs) and to the acidocalcisomes using polyclonal antibodies against the protein and immunoelectron microscopy (91). Downregulation of *TbVtc1* expression by RNAi had marked effects, stopping cell proliferation, changing the morphology and size of acidocalcisomes, and decreasing their V-$H^+$-PPase activity (91). Since the V-$H^+$-PPase generates a protonmotive force that is essential for $Ca^{2+}$ uptake by the $Ca^{2+}/H^+$ counter-transporting ATPase (4, 14) and for polyP synthesis (13), these decreased V-$H^+$-PPase activities would lead to the decrease in $Ca^{2+}$ and short- and long-chain polyP content. PolyP deficiency would then result in a deficient response to hypo-osmotic stress (91)

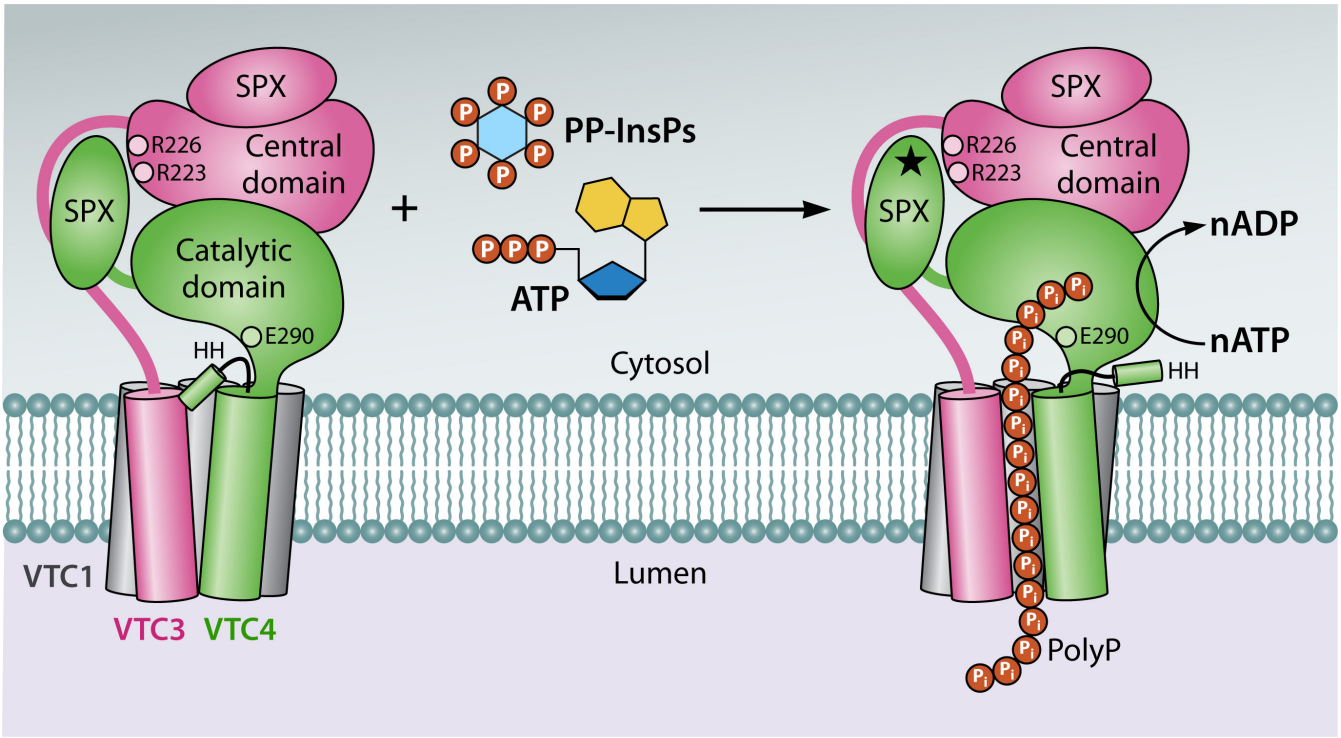


**FIG 5** A model of the activation mechanism of the VTC complex in yeasts. Schematic of the Vtc4/Vtc3/Vtc1 complex. Subunits are colored. The three subunits of VTC1 are shown in gray; VTC3 is in violet; and VTC4 is in green. PP-InsPs bind to the Vtc3 SPX domain. The star indicates the binding resgion of the SPX domains. ATP is used to add a $P_i$ to the polyP chain at the catalytic domain of VTC4. Key amino acids involved are highlighted. HH, horizontal alpha helix fastening the cytosolic entrance of the transmembrane channel for polyP. (Adapted from reference 145 with permission from the authors.)

and perhaps to cytokinesis defects. However, another possible explanation for the cytokinesis defects could be related to the role of the Vtc1 ortholog in *S. pombe* (Nrf1), which is an important regulator of the small Rho-like GTPase Cdc42p (149). This GTPase is found in most eukaryotic cells (150). Cdc42p is important for vacuolar function and morphology, critical for cell polarity and cytokinesis and for docking assembly in *S. cerevisiae* (151, 152).

TbVtc4 also appears to have three transmembrane domains. *In situ* tagged TbVtc4 (92), as well as tagged *T. cruzi* Vtc4 (93), localized to acidocalcisomes, as detected by IFA and immunoelectron microscopy. The catalytic domain of either TbVtc4 or TcVtc4 was able to catalyze the synthesis of short-chain polyP (100–300 $P_i$ units), in contrast to the synthesis of long-chain polyP by the catalytic core of ScVtc4 (92, 93). $PP_i$ inhibited the synthesis of short-chain polyP by TbVtc4 or TcVtc4 and stimulated the synthesis of polyP by ScVtc4 (92, 93). A conditional KO of *TbVtc4* in bloodstream forms resulted in decreased proliferation and short-chain polyP production without affecting long-chain polyP synthesis. These cells had higher sensitivity to hypo-osmotic and hyperosmotic stresses and lower infectivity to animals, but no cytokinesis defects (92). RNAi of *TbVtc4* in procyclic forms also reduced proliferation and short-chain polyP synthesis without affecting long-chain polyP synthesis, suggesting that other polyP polymerases might occur in trypanosomes (93). Interestingly, both TbVtc1 and TbVtc4 appear to be palmitoylated (101).

Variable levels of polyP were observed in different *Leishmania* spp. (148). PolyP was more abundant in late logarithmic growth phase promastigotes of *L. major* and *Leishmania amazonensis* and decreased overtime in stationary phase cultures, although protein Vtc4 levels were constant (148). The role of Vtc4 in *Leishmania* spp. was studied by either knockout or knockdown of its gene expression. PolyP levels were reduced 5- to 10-fold by knockdown of *Vtc4* expression by RNAi in *Leishmania guyanensis*, but this

did not affect mice footpad infection (148). *L. major Vtc4* knockout promastigotes were devoid of short-chain polyP, grew almost normally in culture, and were able to differentiate normally into metacyclic promastigotes (148). Amastigotes had no detectable Vtc4 and only short-chain polyP (148). While late stationary promastigotes were able to infect macrophages and differentiate into amastigotes, they survived less than wild-type parasites (147, 148). When injected into the footpad of mice, they had a delay in replication, but they were still able to produce lesions (148).

*T. gondii* has homologs to Vtc2 and Vtc4. A signature-tagged mutagenesis screen identified a disrupted locus that encodes a protein with homology with yeast Vtc2 (153). The tagged TgVtc2 was shown to have a punctate localization, compatible with acidocalcisomes, and the mutant had a significant reduced level of short- and long-chain polyP, which could be partially restored by complementation with an exogenous gene (153). The mutant tachyzoites were able to differentiate into bradyzoites, and they upregulated sixfold the expression of a FIKK kinase (153). Attempts to knockout *TgVtc2* were unsuccessful, suggesting that the gene is essential. TgVtc4 was shown to co-localize with the V-$H^+$-ATPase to acidocalcisomes (118), but no further studies were reported.

Evidence for the presence of homologs to Vtc1 and Vtc4 in acidocalcisomes of *C. reinhardtii* has also been reported (154). A gene encoding a *ScVtc1* homolog was found to be deleted in a *C. reinhardtii* mutant (*ars76*) with altered ability to acclimate to sulfur deficiency because of its inability to accumulate extracellular or periplasmic arylsulfatase (ARS) (154). The mutant had few acidocalcisomes; was more sensitive to exposure to sulfur-, phosphorus-, or N-deficient conditions; and was defective in trafficking of periplasmic ARS. The phenotypes were complemented by providing an exogenous *CrVtc1* gene (154). A truncated CrVtc4 with the putative kinase domain was expressed and shown to synthesize polyP, while polyP was undetectable in a CrVtc4 loss-of-function mutant (155). Phylogenetic studies revealed that VTC and phosphate transporter genes, like *C. reinhardtii* phosphate transporter C, which transports $P_i$ out of acidocalcisomes, were conserved among species that store phosphorus as vacuolar polyP and absent from genomes of higher plants that store phosphorus as $P_i$ in the vacuoles. This suggests loss of *VTC* and *PTC* genes during evolution to higher plants (155). CrVtc1- and CrVtc4-mediated synthesis of polyP was also measured at different times after adding $P_i$ to P-starved cells, and it was found that their expression was markedly reduced after addition of $P_i$ (156). The authors presented indirect evidence that inositol pyrophosphates could be stimulating polyP synthesis. Addition of $P_i$ together with neomycin, which inhibits inositol phosphate synthesis by binding to phosphatidylinositol 4,5-bisphosphate, accumulated less $P_i$ than controls and had less polyP (156).

Degradation of polyP in the acidocalcisome-like vacuole of *S. cerevisiae* is through the activity of two endopolyphosphatases (they degrade polyP by attacking internal phosphoanhydride bonds): Ppn1 and Ppn2. ScPpn1 was originally described as a homodimer (157) but later found to be a homotetramer of 35-kDa subunits that requires protease activation of a 78-kDa precursor polypeptide (prePpn1) (158). The processed enzyme produces $P_i$ and $polyP_3$ (158). The enzyme requires $Mn^{2+}$ or $Mg^{2+}$; is inhibited by $Ca^{2+}$, $Zn^{2+}$, $P_i$, and $PP_i$; and has a neutral pH optimum (7.5) (157). *Null* mutants in *ScPpn1* are growth defective and accumulate long-chain polyP (159). The production of monomeric $P_i$ suggests that ScPpn1 can also display exopolyphosphatase activity (cleavage of the terminal $P_i$) (158, 160).

ScPpn2 belongs to the phosphoprotein phosphatase family. It has one TMD in the N-terminus and the catalytic domain at its C-terminus, which localizes to the lumen of the acidocalcisome-like vacuole (159–161). ScPpn2 immunoprecipitated from yeast vacuoles had $Zn^{2+}$- or $Co^{2+}$-stimulated endopolyphosphatase activity. Studies with different *ScPpn1*, *ScPpn2*, and *ScVtc* mutants concluded that ScPpn1 might be involved in the mobilization of polyP stores, while ScPpn2 might control polyP chain length and that polyP levels of yeasts are controlled through synthesis rather than degradation (161).

Trypanosomatids have an acidocalcisome protein with homology to ScPpn2, but this enzyme has not been studied (Table 1). In addition, trypanosomatids have an

acidocalcisome vacuolar soluble pyrophosphatase (VSP), which has exopolyphosphatase activity in the presence of $Zn^{2+}$ and pyrophosphatase activity in the presence of $Mg^{2+}$ (99, 109, 162). The enzyme is also present in the cytosol (109). The exopolyphosphatase activity of the *T. brucei* enzyme is inhibited by bisphosphonates, which also inhibit *in vivo* infections (163). The enzyme has a putative calcium EF-hand-binding domain that was originally proposed to be involved in its oligomerization (99), but further structural work failed to find evidence for this effect (164). Downregulation of its expression by RNAi in *T. brucei* bloodstream form (BSF) resulted in decreased levels of short- and long-chain polyP and altered response to phosphate starvation and hypo-osmotic stress (99). Overexpression of the enzyme in *T. cruzi* led to a decrease in $PP_i$ and short- and long-chain polyP, larger acidocalcisomes, defective response to hyperosmotic stress, and lower proliferation in fibroblasts with reduced persistence in tissues of mice (109). The crystal structure of both the *T. brucei* (164, 165) and *T. cruzi* (164) enzyme has been solved and revealed an unusual tetrameric oligomeric state containing head-to-tail dimers (Fig. 6).

Little is known on the mechanisms involved in polyP degradation in acidocalcisomes of other species except for the presence of a putative endopolyphosphatase in acidocalcisomes of the alga *C. merolae* (63).

The conclusion is that acidocalcisomes possess mechanisms for the synthesis (VTC complex) and degradation (endo- and exopoylphosphatases) of polyP. The absence of VTC complex in animal cells and the peculiarities of the polyP-degrading enzymes make them potential drug targets against pathogenic organisms possessing these organelles.

## Channels

The presence of at least three channels has been reported in acidocalcisomes of trypanosomatids, an aquaporin, a potassium channel, and the inositol 1,4,5-trisphosphate receptor ($IP_3R$) (Tables 1 and 2).

*T. cruzi* aquaporin 1 (TcAQP1) co-localized with the V-$H^+$-PPase to acidocalcisomes and the contractile vacuole complex (CVC), as revealed by IFA using polyclonal antibodies against the GFP-tagged protein or against a synthetic C-terminal peptide (105). When expressed in *Xenopus* oocytes, TcAQP1 was shown to be water permeable but not glycerol permeable, in agreement with its phylogenetic analysis that indicates that it belongs to the orthodox (water transporting) aquaporin branch (105). The protein was found to be N-glycosylated (105) and was important for the response of the cells to hypo-osmotic stress (106). Upon hypo-osmotic stress, there was swelling of acidocalcisomes and microtubule- and cyclic AMP-mediated fusion of the organelles to the CVC (Fig. 7) with translocation of TcAQP1 and water release to facilitate cell volume recovery (106). TcAQP1 is also important for the cellular response to hyperosmotic stress as revealed by experiments with inhibitors, or knockdown or overexpressed TcAQP1 (166).

A gene with homology to inward rectifying potassium channels was found in *T. brucei*, and the protein product (TbIRK) was shown to co-localize with V-$H^+$-PPase to acidocalcisomes of procyclic and bloodstream forms (98). The channel was functionally studied by electrophysiology after expressing it in *Xenopus* oocytes and found to be selective for potassium ions and inhibited by cesium but not by barium (98). The sequence TXTGY(F)G of the selectivity filter found in other potassium channels is replaced by the sequence GGYVG in TbIRK, which was confirmed as the selectivity filter by mutagenesis studies (98). Downregulation of its expression in procyclic forms was obtained by RNAi, but no proliferation changes were found.

Early proteomic studies of contractile vacuole fractions of *T. cruzi* reported the presence of peptides from a putative $IP_3R$ (168). The trypanosomatid $IP_3Rs$ possess domains present in other eukaryotes such as the putative suppressor domain-like, ryanodine receptor $IP_3R$ homology (RIH), and RIH-associated domains, and a $Ca^{2+}$-specific selectivity filter, GVGD (169). However, they have five instead of six TMDs and conserve only 4 or 5 of the 10 residues proposed to form a basic pocket that binds $IP_3$ (97). *In situ*

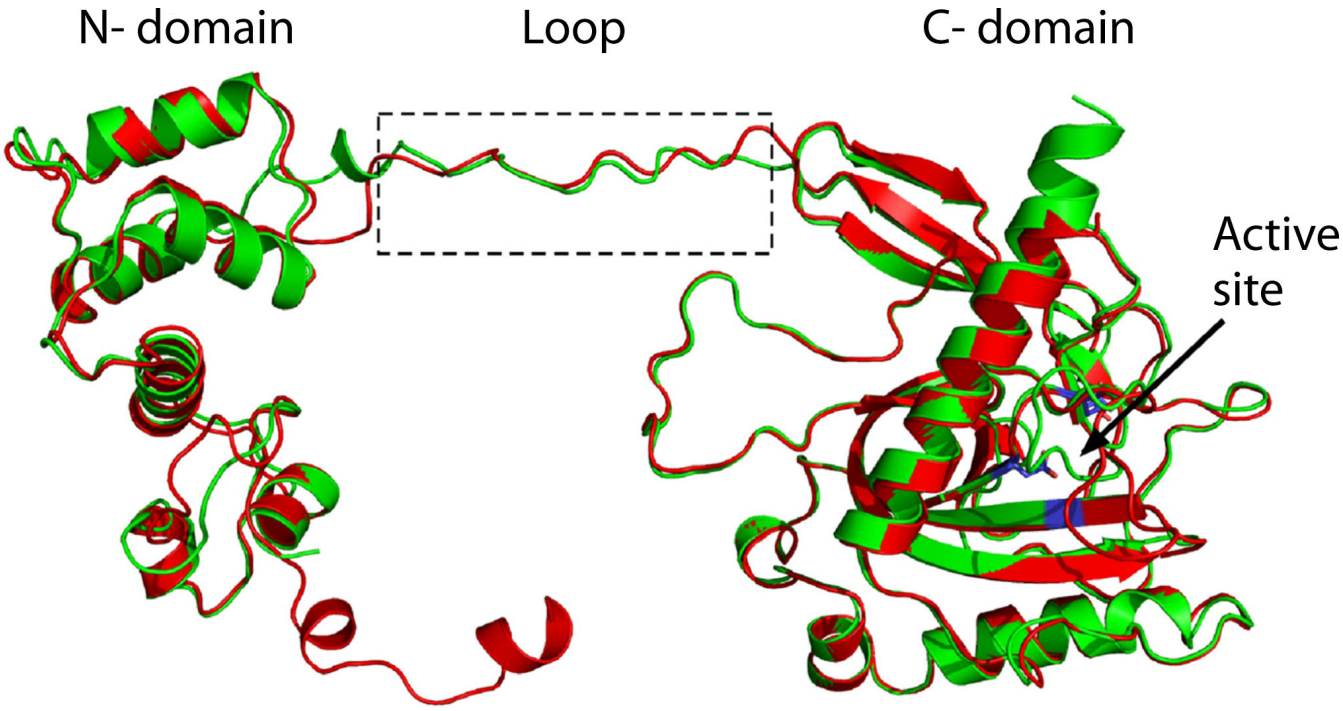


**FIG 6** Structure superimposition of native TcVSP1 (green) and TbVSP1 (red) in the absence of any added substrate or inhibitor ligands. There is an EF-hand N-terminal domain and a C-terminal PPase domain. A long (~17 residues, ~32A) polypeptide chain linker connects the two domains. Blue denotes divalent cation-binding aspartates. (Reproduced from reference 164 with permission from the American Chemical Society.)

tagging of the ortholog gene in *T. brucei* co-localized the protein product with the V-$H^+$-PPase to the acidocalcisomes but not to the ER (97). These results were later confirmed using specific antibodies against *T. brucei* $IP_3R$ ($TbIP_3R$) (26, 170) (Fig. 3). The $TbIP_3R$ function was confirmed by expressing it in DT40 chicken B lymphocytes that are knockout mutants for the three animal $IP_3Rs$ (DT-40–3KO) (171). $IP_3$ was also able to release $Ca^{2+}$ from permeabilized cells, and isolated acidocalcisomes' previous acidification of the organelle by $PP_i$. $Ca^{2+}$ release by uncaging $IP_3$ by UV light was also observed in Fluo4-AM-loaded live procyclic forms (97).

Since acidocalcisomes are rich in phosphorus compounds, the response of $TbIP_3R$ expressed in DT-40–3KO cells to the addition of these compounds to its luminal side was investigated using patch-clamp recordings of nuclear membranes, which are continuous with the ER. $K^+$ at 140 nM was the charge carrier, and $IP_3$ was added to the patch pipette (cytosolic side) (172). The currents generated by $IP_3$ were abolished by acidic pH and heparin and inhibited by 2-APB and caffeine (172). $P_i$ and $PP_i$, added to the luminal side, increased the currents, while $polyP_3$, but no longer-chain polyP, inhibited it. These latter effects were not observed when rat $IP_3R$-1 ($RnIP_3R$) was tested instead of $TbIP_3R$ (172). In summary, the results suggest that $TbIP_3R$ is closed at the acidic conditions of the acidocalcisomes, but alkalinization or polyP hydrolysis favors the channel opening by $IP_3$. Downregulation of *$TbIP_3R$* expression by RNAi showed that it was essential for proliferation and infectivity of *T. brucei* in mice (97).

The *T. cruzi* $IP_3R$ was originally proposed to have an ER localization, although no clear co-localization with *T. brucei* ER marker binding immunoglobulin protein was demonstrated, and the antibodies against the receptor gave a punctate staining in *T. cruzi* (171). Endogenous tagging of $TcIP_3R$ confirmed its acidocalcisome localization (107). Expression of *$TcIP_3R$* in DT4-3KO cells also established its function in releasing $Ca^{2+}$ upon stimulation with $IP_3$ (108, 171).

$TcIP_3R$ single-allele knockout epimastigotes showed reduced proliferation rate and metacyclogenesis, while trypomastigotes had lower $Ca^{2+}$ increase upon attachment to

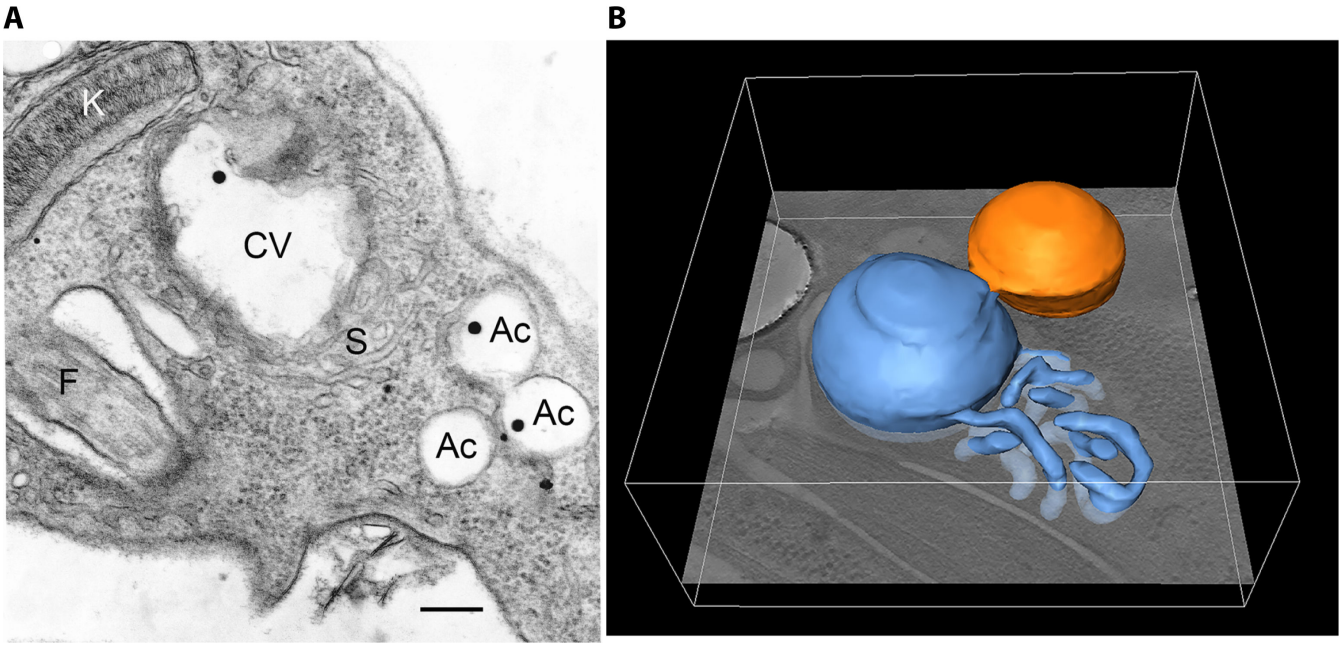


**FIG 7** Contractile vacuole complex of *T. cruzi*. (A) Epimastigote showing the kinetoplast (K), CV, S, F, and similar electron-dense material present in the CV bladder and in Ac. (B) Three dimensional model of a tomogram showing an acidocalcisome (orange) fusing with the CVC (blue) after hypo-osmotic stress. The CVC is represented by a central vacuole or bladder, and tubules or spongiome. Picture taken by Kildare Miranda. Experimental details in reference (167). (Panel A was reproduced from reference 105 with permission from the American Society for Biochemistry and Molecular Biology.) Ac, acidocalcisome; CV, contractile vacuole; CVC, contractile vacuole complex; F, flagellum; S, spongiome.

host cells and were less infective but did not show differences in amastigote replication (171). *TcIP$_3$R* double-allele ablation epimastigotes obtained by CRISPR/Cas9 genome editing also had reduced proliferation but increased metacyclogenesis (108). Similar to the single-allele KO, the double-allele KO trypomastigotes are also less infective without differences in amastigote replication. Single knockout trypomastigotes had a significantly higher transformation rate into amastigotes (171). Conversely, although overexpression of *TcIP$_3$R* in epimastigotes did not modify their proliferation rate, they had reduced metacyclogenesis. Trypomastigotes overexpressing the *TcIP$_3$R* had increased $Ca^{2+}$ levels upon cell attachment and higher infectivity in tissue culture cells (171), without affecting (171) or reducing (108) amastigote replication, and showed a decreased transformation rate into amastigotes (171).

Little is known about the presence of channels in acidocalcisomes of other species. A transient receptor potential (TRP)-like protein is in the acidocalcisome-like vacuoles of *S. cerevisiae* and other fungi (TRPY1 or *yvc1*) (173). This channel is activated by either mechanical force or $Ca^{2+}$ and mediates vacuolar $Ca^{2+}$ release upon hyperosmotic stress (174). The channel has a tetrameric structure displaying activating and inhibiting $Ca^{2+}$-binding sites and co-purifying with an inhibitory phosphatidylinositol 3-phosphate [PI(3)P] lipid (175). A two-pore channel (TDC2) is localized in the acidocalcisome-like platelet dense granules (PDGs) and regulates PDG luminal pH and functions in $Ca^{2+}$ release from PDGs forming perigranular $Ca^{2+}$ microdomains (175).

## Membrane transporters

Several metal ion transporters were identified in trypanosomatid acidocalcisomes. Proteomic analysis of *T. brucei* acidocalcisomes (26) and immunofluorescence analysis of the parasite proteome (89) resulted in the identification of transporters for copper (copper ATPase or TbATP7), zinc (Zn transporters 1 and 2 or TbZnT1 and TbZnT2), and iron or manganese (vacuolar iron transporter or TbVIT) (Table 1).

A zinc transporter (TbZnT1) was identified by proteomic analysis of acidocalcisomes of *T. brucei* (26). TbZnT1 is a member of the cation diffusion facilitator family (CDF) (176). These transporters function as antiporters of $Zn^{2+}$, $Cd^{2+}$, $Co^{2+}$, and/or $Ni^{2+}$ with protons. All contain six transmembrane domains and share characteristic motifs, such as a CDF family-specific signature sequence at the C-terminus (177). A second zinc transporter (TbZnT2) was also found in acidocalcisomes (Table 1). A zinc transporter was also described in acidocalcisomes of *T. cruzi* (Table 2) (103). Zinc has catalyticand structural functions in more than 3,000 human proteins and has also a regulatory function (178). Downregulation of TbZnT1 by RNAi was not lethal in procyclic form or BSF (26), probably because of the presence of other Zn transporters in acidocalcisomes.

A zinc transporter (TgZnT) has also been shown to localize to small vesicles or acidocalcisomes that fuse with the PLVAC in *T. gondii* tachyzoites (113). The protein has six TMDs and is the sole member of the ZnT family of $Zn^{2+}$ transporters. *TgZnT* knockout cells have reduced viability in the presence of extracellular $Zn^{2+}$ (113).

An ortholog to the vacuolar iron transporter (VIT) originally described in *Arabidopsis thaliana* (179) and to the yeast $Ca^{2+}$-sensitive cross-complementer 1 (180) was found in the acidocalcisome proteome of *T. brucei* (TbVIT1) and co-localized with TbV-$H^+$-PPase (26). These transporters are localized to the plant and yeast vacuole, respectively, and have been involved in iron and manganese sequestration into the vacuoles. VIT family members are not found in humans but are found in other fungi, plants, and in malaria parasites, where it apparently localizes to the ER, and its expression downregulation yields reduced liver and blood infections (181). Knockdown of TbVIT1 by RNAi in both procyclic and bloodstream forms resulted in growth defects with a 44% ± 6% and 41% ± 3% reduction in the number of cells 2 and 4 days after tetracycline addition to bloodstream and procyclic forms trypanosomes, respectively, indicating their essentiality for normal growth (26).

A $P_i$ transporter (TbPho91) with homology to *S. cerevisiae* Pho91p, which is a low-affinity sodium-phosphate ($Na^+/P_i$) symporter that was proposed to export $P_i$ and $Na^+$ from the vacuole lumen to the cytosol (182), was localized to acidocalcisomes of *T. brucei* (26, 131). Knockout of *TbPho91* expression affected cell proliferation under phosphate starvation and increased the size of acidocalcisomes (131). Expression of *TbPho91* in *Xenopus laevis* oocytes and two-electrode voltage clamp recordings found that 5-diphosphoinositol pentakisphosphate, an inositol pyrophosphate, stimulated sodium-dependent depolarization of the oocyte membrane potential and $P_i$ conductance. This effect depended on the presence of the Pho91 SPX domain (131). Similar requirement for the SPX domain was found when giant vacuoles of yeasts expressing wild type or SPX mutant symporters from *T. brucei* or yeast were patch-clamped to detect currents (131). The results provided evidence for the role of TbPho91 and Pho91p in $P_i$ and $Na^+$ release from acidocalcisomes to the cytosol and for the role of its SPX domain in mediating its regulation by inositol pyrophosphates.

In contrast with TbPho91, *T. cruzi* Pho91 localizes mainly to the contractile vacuole complex (183). Overexpression of the gene led to higher levels of polyP while its downregulation reduced the growth rate and polyP levels (183).

Other transporters from *T. brucei* like a cation transporter, probably involved in $Mg^{2+}$ uptake, a sulfate transporter, a polyamine transporter, two proteins belonging to the major facilitator superfamily, and a homolog to the multidrug and toxic extrusion protein have not been studied in detail, and their functions are unknown (Table 1).

## Other components

There is physiological evidence for the occurrence of $Ca^{2+}/H^+$ and $Na^+/H^+$ exchange in *T. brucei* procyclic trypomastigotes (184, 185) and *L. donovani* promastigotes (186) acidocalcisomes, but the responsible exchangers have not been identified. Some proteins have been localized to acidocalcisomes of *T. brucei* but have not been studied in detail, such as the putative enzyme acid phosphatase (26), oxidoreductase, endopoly-

phosphatase, and palmitoyl acyl transferase 2 (101), and other proteins like FLA-1 like (102) and STRAP (89) (Table 1).

In *T. cruzi*, the histidine ammonia lyase (HAL) co-localized with the V-$H^+$-PPase to acidocalcisomes, as detected by the epitope tagging the enzyme or with specific antibodies (111). Fusing HAL to the pH sensor pHluorin detected the alkalinization of acidocalcisomes upon addition of histidine, as HAL catalyzes histidine deamination producing ammonia and urocanate (111). The enzyme has five lysine residues in the C-terminal region that are important for its binding to polyP, which inhibits its activity, and for the parasite survival under starvation conditions (111).

## ACIDOCALCISOME BIOGENESIS

Acidocalcisomes share with lysosome-related organelles the transport mechanism of membrane proteins. LROs of animal cells were defined as "cell type-specific modifications of the post-Golgi endomembrane system that have a variety of functions and share some common characteristics with lysosomes" (187). Examples include melanosomes, platelet dense granules, basophil granules, and neutrophil azurophil granules (188).

The first evidence that acidocalcisomes were similar to lysosome-related organelles was provided by studies on the role of adaptor protein 3 (AP-3) complex in the transport of membrane proteins to acidocalcisomes of *L. major* (189). AP complexes mediate vesicular transport of membrane proteins between cellular compartments (190), and AP-3 complex is involved in sorting proteins to lysosome and lysosome-related organelles from the Golgi (191) or endosomes (192). It recognizes its cargo proteins through di-leucine- or tyrosine-based sorting signals (193). The AP-3 complex is a heterotetramer with two large subunits (β3 and δ), one medium subunit (μ) and one small subunit (σ). Deletion of *L. major* AP-3δ subunit did not apparently affect the multivesicular tubule-lysosome of these parasites but resulted in less acidic acidocalcisomes because of lower V-$H^+$-ATPase and V-$H^+$-PPase content and activity (189). The soluble VSP1 (162), however, was not affected and was detectable in mutant and wild-type cells acidocalcisomes. The AP-3δ knockout promastigotes had a lower proliferation rate and were able to differentiate into metacyclic forms and infect macrophages, although they replicated less intracellularly and had attenuated virulence in mice. All these alterations were complemented in a re-expressing cell line (189). These studies were later confirmed in *T. brucei* (100). C-terminal tagged *T. brucei* AP-3β3 and AP-3δ subunits co-localized with V-$H^+$-PPase antibodies to acidocalcisomes, with the Golgi reassembly and stacking protein antibodies to the Golgi and with Rab11 antibodies to recycling endosomes, revealing their dynamic behavior (100). Downregulation of the expression of these adaptins by RNAi resulted in (i) reduction in the proliferation of both procyclic and bloodstream forms; (ii) progressive reduction in the number and calcium and phosphorus content of acidocalcisomes, leading to their disappearance; (iii) lack of detection of the V-$H^+$-PPase by IFA; (iv) a considerable reduction in acidic $Ca^{2+}$ and in total levels of $PP_i$ and polyP; and (v) reduced volume recovery after hypo-osmotic or hyperosmotic stress, compared with control cells (100). As occurred in *L. major* (189), trafficking of proteins (membrane glycoprotein p67 and luminal cathepsin L) to the lysosome was unaffected, and the mutant parasites were less virulent in mice (100).

Interestingly, mutation of AP-3 subunits in humans causes the Hermansky-Pudlack syndrome, characterized by decreased acidocalcisome-like platelet dense granules, which contain polyP (24), and resulting in bleeding problems (194). Mutations in *ALP5* (AP-3δ) or in *APS3* (AP-3σ) in *S. cerevisiae* also resulted in decreased polyP accumulation in the acidocalcisome-like vacuoles associated to mistargeting of the Vtc5 subunit of the VTC complex (195).

In addition of the evidence of the role of AP-3 complex in the transfer of membrane proteins to acidocalcisomes and acidocalcisome-like organelles like platelet dense granules and other LROs (196), the organelles were identified during assembly in the *trans*-Golgi in a variety of algae and protists (41). Small vesicles were observed fusing with the organelle membrane of *C. reinhardtii*, *C. monoica*, and *T. brucei*, in a manner

similar to those of some lysosome-related organelles (197) providing support for their *trans*-Golgi origin (Fig. 8).

Expression downregulation of several proteins has also been reported to affect the biogenesis of acidocalcisomes in trypanosomatids. *Null* mutants of the first enzyme in the sphingolipid synthesis pathway (serine palmitoyl transferase) of *L. major* have morphologically altered acidocalcisomes with lower long-chain polyP content (198). Acidocalcisomes of *null* mutants for target of rapamycin 3 (TOR 3) kinase of *L. major* are smaller and less numerous with little DAPI staining of polyP, suggesting a lower polyP content (199). In contrast, RNAi of TOR three kinase in *T. brucei* resulted in larger acidocalcisomes with increased levels of $PP_i$ and polyP (200). In addition, RNAi downregulation of a motor kinesin of *T. brucei* resulted in functional alterations (less $Ca^{2+}$ release), suggesting that this motor protein could be involved in the traffic of vesicles to acidocalcisomes (201).

In conclusion, beyond the role of the AP-3 complex in the targeting of proteins to the acidocalcisomes, the knowledge is still incomplete because there are probably other trafficking proteins involved in the biogenesis of the organelle. No targeting signals of proteins localized to the acidocalcisomes, besides the presence of one or more tyrosine-based sorting signals with the YXXØ (Ø corresponds to a hydrophobic amino acid) consensus motif (26), have been described.

## INTERACTION OF ACIDOCALCISOMES WITH OTHER ORGANELLES

Two types of interactions between acidocalcisomes and other organelles have been reported. The first one is through membrane fusion with the CVC that occurs in *T. cruzi* (167) and potentially in other species possessing acidocalcisomes and a CVC. The second is through membrane contact sites, which are regions at which organelles interact to exchange biomolecules and that are usually less than 30 nanometers apart (202).

Video microscopy of *T. cruzi* epimastigotes submitted to hypo-osmotic stress showed vesicle fusion events both with the contractile vacuole and between vesicles (106). GFP-tagged TcAQP1 labeled the acidocalcisomes, but during hypo-osmotic stress, most of the label was translocated to the contractile vacuole (106). It was possible to quantify the translocation by counting the cells with one bright spot corresponding to the CVC since labeling of acidocalcisomes faded with time. Under isosmotic conditions, this translocation of TcAQP1 was stimulated by cyclic AMP analogs and cyclic adenosine monophosphate (cAMP) phosphodiesterase inhibitors, while preincubation with cyclic AMP or microtubule inhibitors decreased translocation under hypo-osmotic stress (106). The results are consistent with microtubule- and cyclic AMP-dependent traffic of acidocalcisomes toward the CVC culminating in fusion of the organelles. Further evidence of fusion of acidocalcisomes and the CVC was provided by the change in localization of GFP-tagged Rab32 or VAMP7 from acidocalcisomes to the CVC under hypo-osmotic stress and electron tomography evidence of fusion of the organelles (167) (Fig. 7B). Acidocalcisomes from *C. reinhardtii* (18) and *D. discoideum* (17) also appear to contact their CVCs under hypo-osmotic conditions.

In addition to fusion with the CVC, there is also evidence of fusion of acidocalcisomes between themselves decreasing their numbers and becoming larger, for example, upon hypo-osmotic stress (17, 106) or after knockdown of AP-3 subunits (100) or Vtc1 (91).

Acidocalcisomes of trypanosomatids also interact with other organelles, such as mitochondria, nuclei, and lipid inclusions, through membrane contact sites (75). The interaction with mitochondria has been studied in more detail in *T. brucei*. In animal cells, the close apposition of the endoplasmic reticulum to the mitochondria facilitates $IP_3R$-dependent $Ca^{2+}$ transfer, which is important to maintain the mitochondrial bioenergetics (203). A similar situation occurs between acidocalcisomes, where the $IP_3R$ localizes in trypanosomatids (97, 107), and the mitochondria, as demonstrated by super-resolution structured illumination microscopy, electron microscopy, proximity ligation assays, and functional studies (202). Super-resolution and electron microscopy revealed that the two organelles appear to be less than 30 nm apart, and proximity ligation assays using

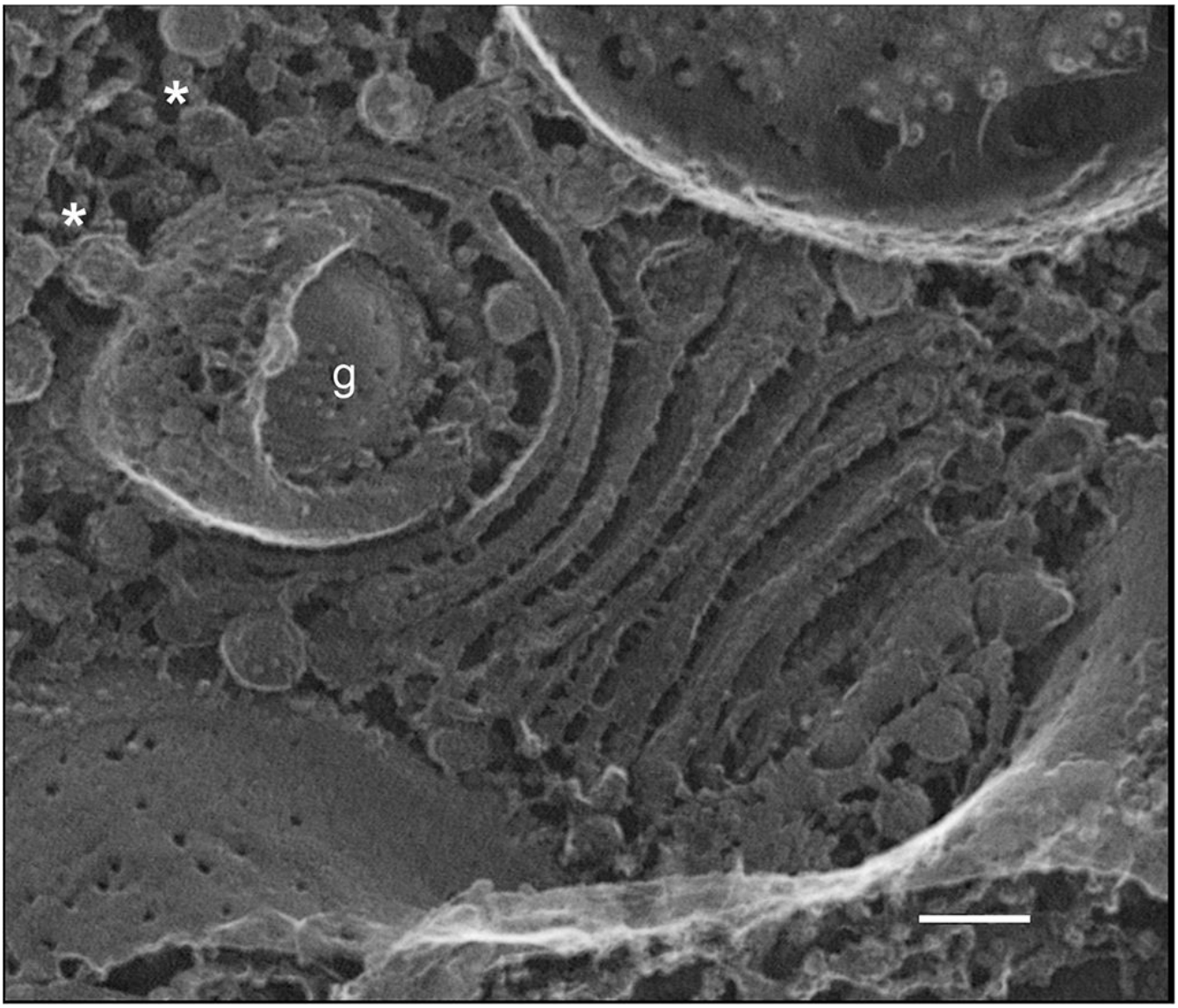


**FIG 8** Acidocalcisomes from *C. reinhardtii* assembling at the *trans*-Golgi face. Note the rugose E-face with granule and fusing Golgi vesicles (asterisks); Bar, 100 nm. (Reproduced from reference 41with permission from Elsevier.) g, polyphosphate granule.

antibodies against tagged acidocalcisome TbIP$_3$R and mitochondrial voltage dependent anion channel (VDAC) detected several contacts between the organelles (202). In addition, downregulation of the TcIP$_3$R expression resulted in alterations in the mitochondrial bioenergetics (108).

## ROLE IN CALCIUM SIGNALING

$Ca^{2+}$ signaling is the result of cytosolic $Ca^{2+}$ increase due to $Ca^{2+}$ release from intracellular stores or $Ca^{2+}$ influx through plasma membrane channels and regulates numerous cellular processes including proliferation, differentiation, and cellular motility. In trypanosomatids, acidocalcisomes are major $Ca^{2+}$ stores and possess mechanisms for $Ca^{2+}$ uptake ($Ca^{2+}$-ATPases) and $Ca^{2+}$ release ($IP_3R$). In other species, acidocalcisomes and acidocalcisome-like structures have been shown to possess $Ca^{2+}$-ATPases but, except for the acidocalcisome-like vacuole of *S. cerevisiae* that possesses a $Ca^{2+}$ release mechanism ($Ca^{2+}/H^+$ exchanger VCX1) (204), it is not known how $Ca^{2+}$ release occurs and whether it is involved in signaling.

Two important functions have been attributed to the acidocalcisome releasable $Ca^{2+}$ in *T. cruzi*. One is the regulation of mitochondrial bioenergetics by the $TcIP_3R$-mediated acidocalcisome $Ca^{2+}$ release (28) (Fig. 9). $Ca^{2+}$ is taken up by the mitochondria and results in activation of pyruvate dehydrogenase dephosphorylation and increased $O_2$ consumption. In the absence of $TcIP_3R$, epimastigotes were viable but showed slower proliferation rate and increased metacyclogenesis (108). Trypomastigotes were less infective, and amastigotes did not show changes in replication. In addition to lower $O_2$ consumption in the *null* mutants, there was an increased phosphorylation of pyruvate dehydrogenase E1a subunit, AMP:ATP ratio, ammonia production, and autophagy (108). Overexpression of $TcIP_3R$ led to lower metacyclogenesis, host cell invasion, and amastigote replication (108).

Acidocalcisome releasable $Ca^{2+}$ is also important for host cell invasion by *T. cruzi*. There is an increase in the parasite cytosolic $Ca^{2+}$ upon its attachment to host cell that is needed for normal invasion (205, 206). That this $Ca^{2+}$ increase is coming from the acidocalcisomes is supported by the deficient invasion when the acidocalcisome *TcIP$_3$R* expression is downregulated (108) or when the phospholipase C, which generates $IP_3$, is inhibited (207). Host cell invasion requires energy (208), and $TcIP_3R$-dependent acidocalcisome $Ca^{2+}$ release is important to maintain mitochondrial bioenergetics (108). Finally, depletion of acidocalcisome $Ca^{2+}$ by treatment of the cells with a combination of ionomycin and nigericin inhibits host cell invasion (207).

## ROLE IN OSMOREGULATION

This role has been studied mainly in trypanosomatids. Trypanosomatids are submitted to drastic fluctuations in osmolarity during their life cycles. In the insect vector lower digestive tract, epimastigotes of *T. cruzi* are submitted to increasing osmolarities that reach values of up to 1,000 mosmol/kg in the yellow rectal content (209). When the blood stages of trypanosomatids circulate through the kidney of their mammalian hosts, they need to resist up to 1,200–1,400 mosmol/kg in the ascending limb of the vasa recta and return to isosmotic conditions of 300 mosmol/kg a few seconds later (210). In addition, these parasites, like all cells, need to regulate their volumes continuously (28).

As described above (Interaction of Acidocalcisomes with Other Organelles), upon hypo-osmotic stress, acidocalcisomes of *T. cruzi* take part in a complex pathway that leads to their fusion with the contractile vacuole complex and transfer of TcAQP1 (106). A model was proposed (106) suggesting that cell swelling, by activating a mechanosensitive channel, causes an increase in cAMP resulting in a microtubule-dependent movement and fusion of acidocalcisomes with the contractile vacuole and translocation of TcAQP1. This fusion would also lead to transfer of osmolytes (cations and phosphorus resulting from the hydrolysis of polyP) to the CVC, leading to water uptake that could then be released out of the cells. The process would be terminated by a phosphodiesterase that hydrolyzes cAMP to 5′-AMP. Some evidence in favor of this model is that (i) a mechanosensitive channel (211), adenylyl cyclases (212), and a cAMP phosphodiesterase C (213) are present in the CVC; (ii) fusion of acidocalcisomes to the CVC is enhanced by cAMP analogs and PDE inhibitors and inhibited by microtubule and adenylyl cyclase inhibitors (106); and (iii) there is hydrolysis of polyP upon hypo-osmotic stress (13) (Fig. 10).

The involvement of acidocalcisomes in the response to hypo-osmotic stress has also been reported in other trypanosomatids. *L. major* acidocalcisomes were reported to lose $Na^+$ and $Cl^-$ (214), and *T. brucei* acidocalcisome V-$H^+$-PPase downregulation led to a deficient regulatory volume decrease after hypo-osmotic stress (127).

On the other hand, hyperosmotic stress results in increased synthesis of acidocalcisome polyP (13), which could have a role in sequestering inorganic ions. This sequestration would reduce the ionic strength increased by water elimination and prevent cell damage (166, 215).

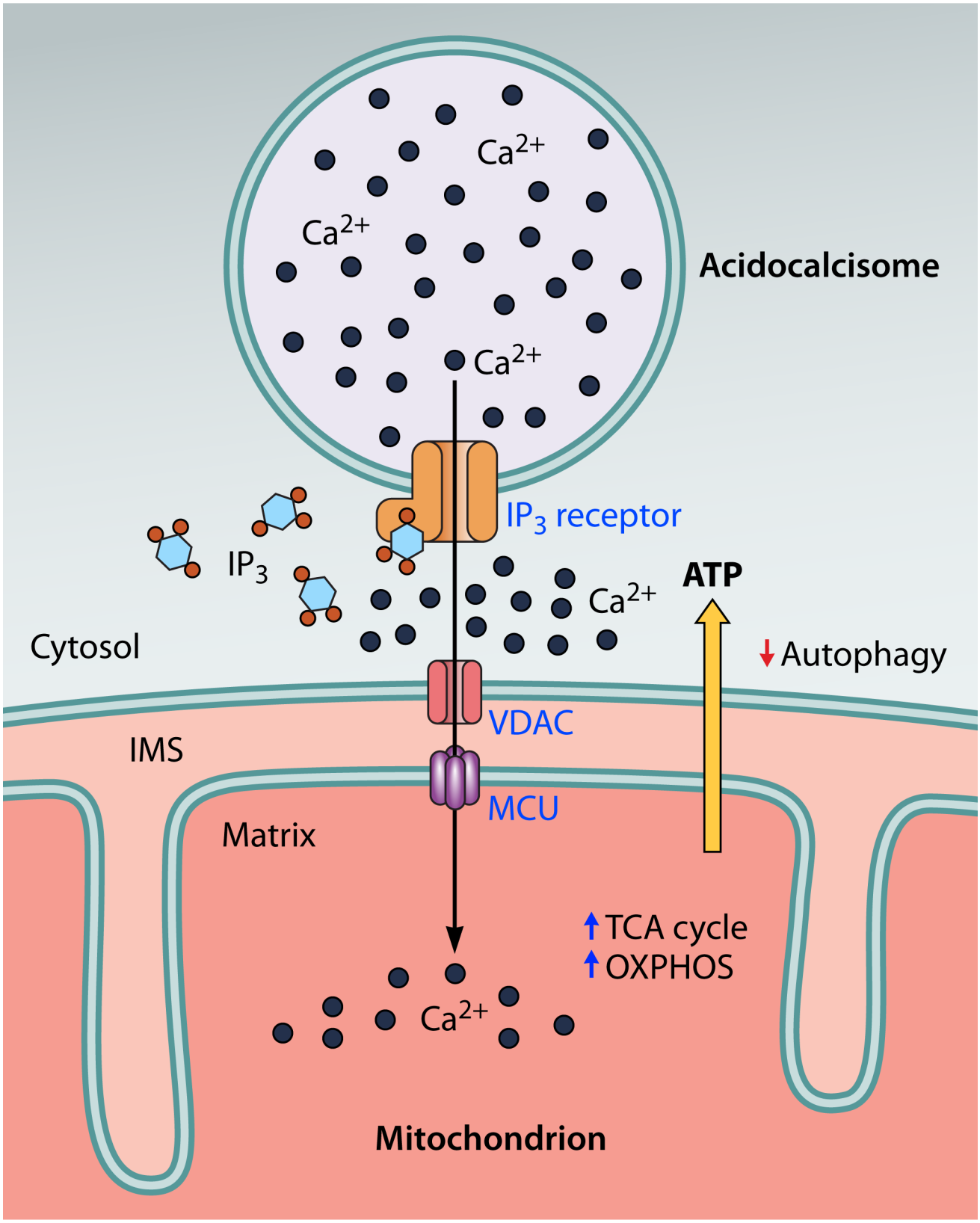


**FIG 9** Acidocalcisomes are in close contact with the mitochondria. $Ca^{2+}$ (black circles) is released from acidocalcisomes upon stimulation of the $IP_3$ receptor ($IP_3R$) by $IP_3$, and after passing the outer mitochondrial membrane through the highly permeable VDAC, it is handled by the MCU. When in the matrix, $Ca^{2+}$ stimulates the TCA function and oxidative phosphorylation (OXPHOS) with the generation of ATP, preventing autophagy. MCU, mitochondrial $Ca^{2+}$ uniporter; VDAC, voltage-dependent anion channel.

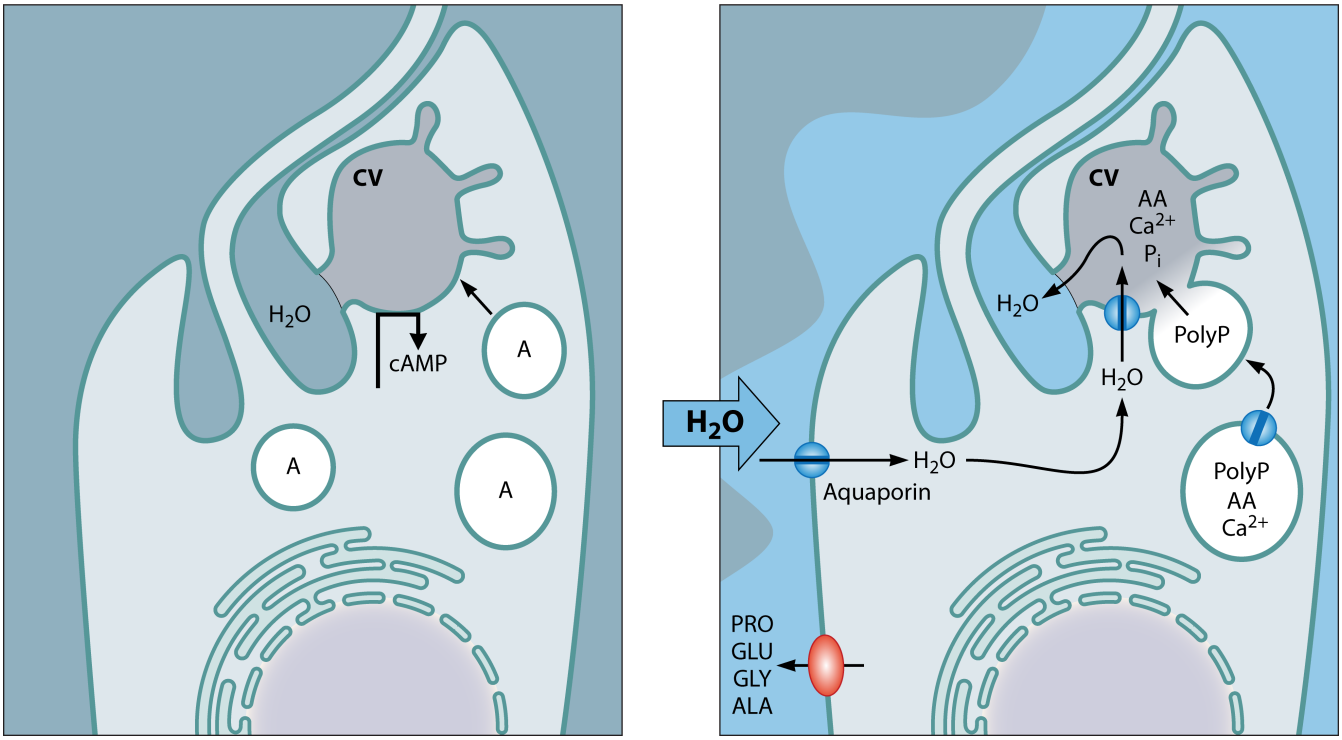


**FIG 10** Model proposed for regulatory volume decrease in *T. cruzi*. Cell swelling causes activation of an adenylyl cyclase, probably by a mechanosensitive channel. cAMP formed stimulates the microtubule-dependent fusion of acidocalcisomes with the contractile vacuole and translocation of an aquaporin. A rise in ammonia in acidocalcisomes activates an exopolyphosphatase activity, which cleaves polyP, releasing inorganic phosphate residues and polyP-chelated cations. The resulting osmotic gradient sequesters water through the aid of the aquaporin. Water is released into the flagellar pocket. Amino acid release contributes to the volume recovery. A, acidocalcisome, AA, amino acid; CV, contractile vacuole.

## ROLE IN CATION AND PHOSPHORUS STORAGE

Acidocalcisomes store phosphorous compounds such as $P_i$, $PP_i$, and polyP, combined with inorganic ($Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $K^+$, and $Na^+$) and organic cations, like lysine, arginine, ornithine, and polyamines. Except for the acidocalcisome-like platelet dense granules (24), or the secretory granules of mast cells (25), which can release the intact polyP polymer, phosphorus release from most acidocalcisomes is in the form of orthophosphate and through $P_i$ transporters like TbPho91 and *S. cerevisiae* Pho91p (131). *Dictyostelium discoideum* also releases polyP (~9 mer) (216), but, although their acidocalcisome-like mass dense granules are rich in polyP (17), it is not known whether they are the source of extracellular polyP.

PolyP release in the case of human platelets has been extensively studied since it was found to have roles in blood clotting and fibrinolysis (217). Platelet-size polyP (~60 to 100 mer) accelerates blood clotting by activating the contact pathway, promoting factor V activation, and inhibiting the function of tissue factor pathway inhibitor (TFTPI). It was found that platelet-size polyP accelerates factor V activation by factor Xa, thrombin (217, 218), and factor XIa (219) and promotes factor XI back-activation by thrombin (220). It also delays clot lysis by promoting the thrombin-activatable fibrinolysis inhibitor (217) (Fig. 11). The ability of polyP to activate the contact pathway was shown to depend on the polymer length being more relevant with long-chain polyP, like that present in bacteria (218). Long-chain polyP found in bacteria is a potent proinflammatory agent (221), suppresses complement (222), and modulates fibrin clot structure and stability (223). PolyP secreted from mast cells could be important for their proinflammatory and procoagulant activity (25).

Storage of polyP in acidocalcisomes could be important as a phosphorus reserve for the synthesis of nucleic acids and phospholipids when the cells multiply. *T. cruzi*

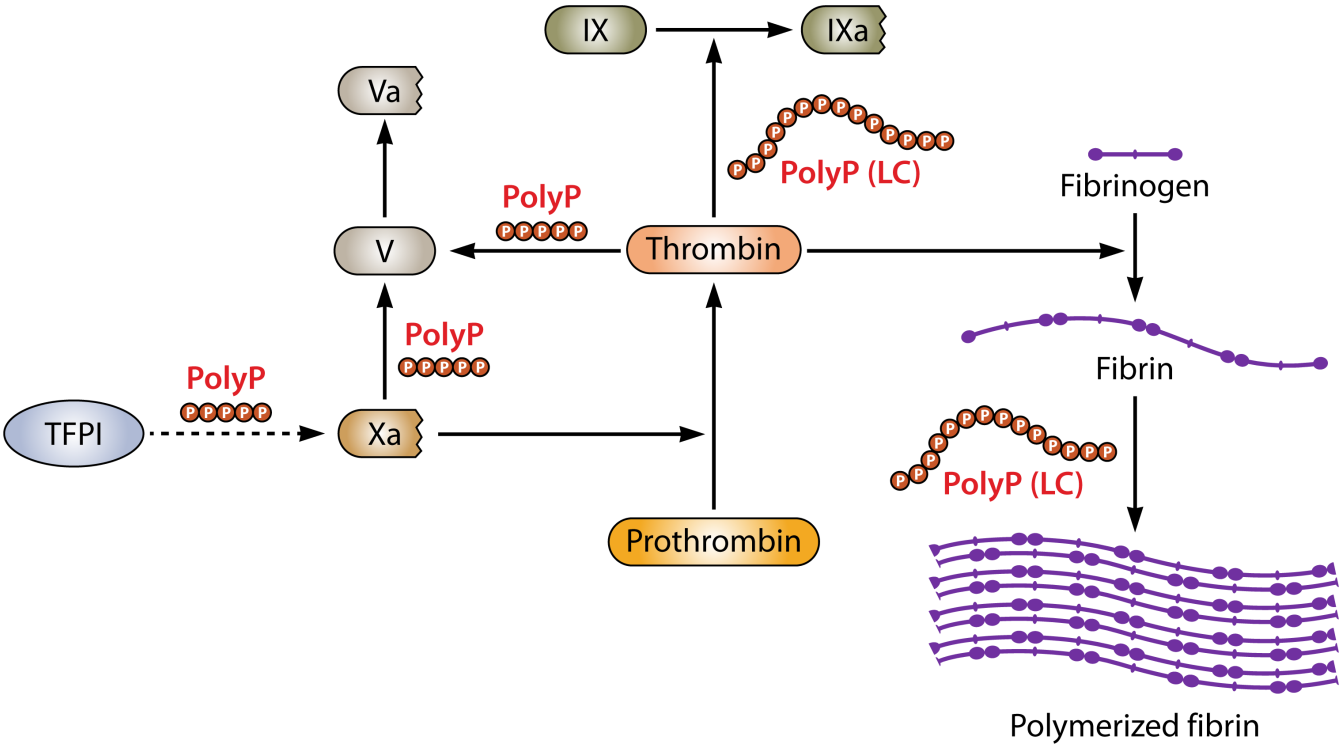


**FIG 11** Steps of the coagulation cascade affected by polyP. PolyP (red) accelerates factor V activation by factors Xa and thrombin, accelerates factor IX back-activation by thrombin, inhibits the ability of TFPI to inhibit factor Xa, and enhances fibrin polymerization. LC, long-chain polyP; polyP, polyphosphate.

epimastigotes increase their synthesis of short- and long-chain polyP during the lag phase of proliferation and then consume it when they start dividing (13). PolyP is also higher during the logarithmic phase of growth and decreases in *Leishmania* spp. during stationary phase (148). There is also an increase in polyP synthesis when trypomastigotes differentiate into amastigotes, which are the forms that multiply within the host cells and possibly need to store polyP for their rapid intracellular replication (13). PolyP hydrolysis and synthesis also occurs under hypo-osmotic and hyperosmotic stress, respectively, and phosphorus and cations could serve as osmolytes with a role in cell recovery after osmotic stress (215). Finally, polyP could serve as chelating agent for inorganic and organic cations allowing their safe storage within acidocalcisomes. Some of these cations are essential for cell growth as they are required for a variety of reactions. For example, in trypanosomes zinc is a catalytic/structural cofactor of many metalloproteins; copper is a cofactor for Cu-containing enzymes, such as cytochrome c oxidase; iron is a cofactor of important enzymes, like iron superoxide dismutases (224); and manganese is required for protein glycosylation in the Golgi complex (225). These nutrient metal ions are concentrated for eventual delivery to the cytosol or other organelles. However, these metals are deleterious if they achieve high concentrations in the cell cytosol. In some cases, like with iron and copper, these metals can generate reactive oxygen species via Fenton-based reactions (226). In other cases, like with zinc and manganese, metabolic enzymes can be inhibited (227). Sequestration of these metals within acidocalcisomes could prevent their deleterious cellular reactions. This sequestration is driven by the pH gradient with the cytosol as many of these transporters take up metals in exchange for protons, which are taken up by the vacuolar $H^+$-ATPase (V-ATPase) or the vacuolar $H^+$-pyrophosphatase (VP1) localized to acidocalcisomes. The complexes of these metals with polyP within acidocalcisomes would help to prevent their release.

Except for $Ca^{2+}$, for which there is a specialized channel, little is known on how other inorganic and organic cations are released from acidocalcisomes.

## OTHER ROLES OF ACIDOCALCISOMES

Other roles of acidocalcisomes include their involvement in autophagy, regulation of pH homeostasis, and infectivity.

The finding that blocking acidocalcisome biogenesis in *T. brucei* by downregulation of the expression of δ and β3 subunits of the AP-3 complex by RNAi inhibited amino acid starvation-induced autophagy revealed the involvement of acidocalcisomes in the initiation of the autophagic process (228). There was a correlation between acidocalcisome acidification and autophagy. Acidocalcisome acidification increased when autophagy was induced by starvation or vanadate treatment, while acidocalcisome alkalinization by bafilomycin $A_1$, monensin, or ionomycin and $NH_4Cl$ treatment inhibited autophagy (228). Notably, RNAi depletion of neither the V-$H^+$-ATPase nor the V-$H^+$-PPase, which acidify the acidocalcisomes, showed inhibition of autophagy. Phosphatidylinositol 3-phosphate associates with acidocalcisomes upon starvation and could be involved in autophagy initiation (228).

It was found that when the expression of the V-$H^+$-PPase of *T. brucei* procyclic forms was downregulated, the cells recovered their intracellular pH after acidification at a slower rate than controls and to a more acidic final pH, suggesting the involvement of these acidic compartment in pH homeostasis (127).

There is indirect evidence that acidocalcisome polyP is important for infectivity. Expression downregulation of acidocalcisome enzymes like the V-$H^+$-PPase (127) or Vtc4 (92), the catalytic subunit of the VTC complex in *T. brucei*, decreased polyP levels and virulence in mice. Expression downregulation of other proteins involved in the biogenesis of acidocalcisomes such as the AP-3 complex subunits in *T. brucei* (100) and the TOR kinase three in *L. major* (199) also reduces parasite virulence. Similarly, overexpression of the acidocalcisome-located VSP in *T. cruzi* (109) makes the parasite less prone to cause a persistent mouse infection.

## ACIDOCALCISOMES AS DRUG TARGETS

The presence and relevance of acidocalcisomes in pathogenic eukaryotes suggest that they can be drug targets (229). Several acidocalcisome components are present in pathogens but absent in animals, like the V-$H^+$-PPase (9, 14, 20, 50, 115, 120, 127, 128), VTC complex (91–93, 148, 153), Pho91 (131), and VIT (26), while others are distantly related to those present in animals, like VSP (99, 163, 164), V-$H^+$-ATPase (117, 118), and $Ca^{2+}$-ATPase (96, 104). Some of these pumps and enzymes have been screened for potential inhibitors (230). For example, the V-$H^+$-PPase from plants is inhibited by the pyrophosphate analogs known as bisphosphonates, which contain a non-hydolizable P-C-P instead of a P-O-P backbone (231). AMDP and IDP, which have a non-hydrolyzable P-N-P group, are currently used as *in vitro* inhibitors of the enzyme (232). AMDP was shown to inhibit intracellular replication of *T. gondii* without affecting host infection (50, 233). The VSP present in trypanosomatids (99, 162) is essential for their normal proliferation and is inhibited by bisphosphonates (163), some of which are active in mice infections by *T. brucei* (99). Some drugs, like diamidines (234), accumulate in acidocalcisomes, although the involvement of this phenomenon in their mode of action is not known.

In summary, there is convincing evidence that acidocalcisomes are potential drug targets and that continuing their study could contribute to new approaches for chemotherapy of protozoan parasite infections.

## CONCLUSIONS AND FUTURE DIRECTIONS

Significant progress has been made in understanding the structure and function of acidocalcisomes in several species. Acidocalcisomes and acidocalcisome-like organelles have been identified in most phylogenetic groups. The search for acidocalcisome-like organelles led to the unexpected finding of polyP in human platelet dense granules (24) and mast cell granules (25) and its secretion, which led to the discovery of the role of this polymer in blood coagulation (217), thrombosis, and inflammation (235). Electron

microscopy studies have been fundamental for understanding their ultrastructure (41) and the elemental organization within the organelle (78). Progress has been made in the characterization of the channels, pumps, transporters, and enzymes of these organelles (26) and in their chemical composition (88).These studies led to the discovery in eukaryotes of enzymes previously found only in plants and archaea (V-$H^+$-PPase) (9) and to the first eukaryotic enzymes involved in the synthesis of polyP (VTC complex) (142). *Chlamydomonas* acidocalcisomes have been developed as models for the study of organellar metal acquisition (85). Trypanosomatids and yeasts have been especially useful for advancing our understanding of the function of their different components and their involvement in cell signaling. The regulation of polyP synthesis in the acidocalcisome-like organelles of yeasts (143) and of the $P_i$ release from acidocalcisomes of trypanosomes (131) by inositol pyrophosphates was first investigated. The finding of mechanisms for $Ca^{2+}$ uptake and release in acidocalcisomes resulted in the discovery of the role of these organelles rather than the endoplasmic reticulum in the regulation of mitochondrial bioenergetics in trypanosomatids (108). A novel role in osmoregulation involving fusion of acidocalcisomes with the contractile vacuole of *T. cruzi* (106), potentially present in other species possessing these organelles, was described. Future studies should include the investigation of (i) the role of the enzymes and transporters newly identified; (ii) the reason for organic cation accumulation and whether polyamines are present in all acidocalcisomes; (iii) the tethers that link acidocalcisomes to other organelles; (iv) whether the membrane contact sites are used for transfer of polyP or cations between organelles; and (v) additional roles of acidocalcisomes in cell signaling. In addition, it would be interesting to investigate whether some acidocalcisome-like vacuoles present in ciliates, other lysosome-related organelles of animal species, or organelles accumulating metals, such as the zincosomes (236), possess polyP.

## ACKNOWLEDGMENTS

We thank Richard J. Wheeler (University of Oxford) for providing the list of putative acidocalcisome proteins identified by the TrypTag project in *Trypanosoma* brucei and Ursula Goodenough (Washington University) for useful comments.

Cited work from this laboratory and preparation of this review were supported by grant AI173402 from the National Institute of Allergy and Infectious Diseases. R.D. is the Barbara and Sanford Orkin/Georgia Research Alliance Eminent Scholar.

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## FUNDING



| Funder | Grant(s) | Author(s) |
|---|---|---|
| HHS \| NIH \| National Institute of Allergy and Infectious Diseases (NIAID) | AI173402 | Roberto Docampo |



## AUTHOR CONTRIBUTIONS

Roberto Docampo, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing

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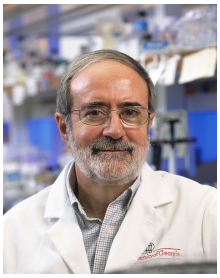

**Roberto Docampo** received an MD in 1972 from the University of Buenos Aires, a PhD in Microbiology in 1977 from the Federal University of Rio de Janeiro (UFRJ), and a PhD in Biological Chemistry in 1979 from the University of Buenos Aires. He then spent four years as a Visiting Scientist at the NIH before joining first, the UFRJ, and then the Rockefeller University, as Visiting Professor. He joined the University of Illinois at Urbana-Champaign in 1990 and in 2005 he moved to the University of Georgia where he remains as Distinguished Research Professor of Cellular Biology and Eminent Scholar. Docampo is best known for his work on the discovery and characterization of acidocalcisomes of different species. His lab was the first to determine the presence and secretion of polyphosphate by human platelets, which led to the discovery of its role in blood clotting. Docampo is Fellow of the American Academy of Microbiology and of the American Association for the Advancement of Science.