Acidocalcisomes Are Functionally Linked to the Contractile Vacuole of Dictyostelium discoideum*

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The mass-dense granules of Dictyostelium discoideum were shown to contain large amounts of phosphorus, magnesium, and calcium, as determined by x-ray microanalysis, either in situ or when purified using iodixanol gradient centrifugation. The high phosphorus content was due to the presence of pyrophosphate and polyphosphate, which were also present in the contractile vacuoles. Both organelles also possessed a vacuolar H\(^+\)/H\(_{11001}\)PPase, an H\(^+\)-pyrophosphatase, and a Ca\(^{2+}\)-ATPase, as determined by biochemical methods or by immunofluorescence microscopy. The H\(^+\)-pyrophosphatase activity of isolated mass-dense granules was stimulated by potassium ions and inhibited by the pyrophosphate analogs aminomethylenediphosphonate and imidodiphosphate and by KF and N-ethylmaleimide in a dose-dependent manner. The mass-dense granules and the contractile vacuole appeared to contact each other when the cells were submitted to hyposmotic stress. Acetazolamide inhibited the carbonic anhydrase activity of the contractile vacuoles and prolonged their contraction cycle in a dose-dependent manner. Similar effects were observed with the anion exchanger inhibitor 4,4′-disothiocyanatodihydrostilbene-2,2′-disulfonic acid and the vacuolar H\(^+\)-ATPase inhibitor bafilomycin A\(_1\). Together, these results suggest that the mass-dense granules of D. discoideum are homologous to the acidocalcisomes described in protozoan parasites and are linked to the function of the contractile vacuole.

 Ionic calcium is involved in the regulation of several biological processes. In mammalian cells, Ca\(^{2+}\) homeostasis is regulated by the concerted operation of several pumps and exchangers located in the plasma membrane, mitochondria, and endoplasmic reticulum (1). In contrast to most mammalian cells, in the slime mold Dictyostelium discoideum, there are two non-mitochondrial Ca\(^{2+}\) stores, one that is sensitive to the second messenger inositol 1,4,5-trisphosphate (possibly the endoplasmic reticulum) (2) and another that is acidic (3, 4). Evidence has accumulated that this acidic compartment is related to the contractile vacuole complex of D. discoideum. Initial studies using a \(^{45}\)Ca\(^{2+}\) uptake assay and isolated cell fractions identified a major ATP-dependent Ca\(^{2+}\) transport system associated with intracellular vesicles (5). Subsequent studies revealed that these vesicles fractionate with vacuolar H\(^+\)-ATPase (V-H\(^+\)-ATPase)\(^1\)-containing vacuoles (3, 6) named acidosomes (7). Acidosomes were postulated to be part of the spongione of the contractile vacuole complex (8) or fragmented contractile vacuole membranes (9). The Ca\(^{2+}\) transport activity of these vacuoles was shown to be vanadate-sensitive (5), thapsigargin-insensitive (6), and facilitated by the elevated intravesicular proton concentration (3, 6, 10). Furthermore, a gene encoding a plasma membrane-type calcium ATPase (PMCA) Ca\(^{2+}\)-ATPase (pat1) was cloned, and its protein product (PAT1) was found to coco-localize with bound calmodulin to membranes of the contractile vacuole (11, 12).

In addition to these biochemical studies, mass-dense granules containing large amounts of calcium together with phosphorus were found in freeze-dried cryosections of rapid-frozen D. discoideum amebas using energy-dispersive x-ray microanalysis (13, 14). These granules are similar in their chemical composition to the polyphosphate bodies described in many microorganisms (15, 16), including D. discoideum (17), and to the more recently described acidocalcisomes of trypanosomatids and apicomplexan parasites (18, 19). In trypanosomatids and apicomplexan parasites, the acidocalcisomes have also been shown to contain proton and calcium pumps and several exchangers in their limiting membranes (20–27). However, the presence of such transporters has not been investigated in the mass-dense granules or polyphosphate bodies of D. discoideum, and the relationship between these granules, the acidosomes, and the contractile vacuole complex of D. discoideum remains undefined. One of the pumps present in the acidocalcisomes of parasitic protozoa is the H\(^+\)-pyrophosphatase (H\(^+\)-PPase). H\(^+\)-PPases have also been described in plants, algae, and bacteria (28, 29). A pyrophosphate activity was detected in total membrane extracts of D. discoideum (30), although its subcellular localization was not investigated.

We report here the isolation of the mass-dense granules of D. discoideum and provide evidence for the presence of several pumps in their limiting membranes. An H\(^+\)-PPase with characteristics similar to those of the plant and acidocalcisomal enzyme was located by biochemical and immunological techniques in these organelles and in the contractile vacuoles, where it co-locализes with the V-H\(^+\)-ATPase and the Ca\(^{2+}\)-ATPase PAT1. Polyphosphate was also detected biochemically and by 4′,6-diamidino-2-phenylindole (DAPI) staining in both the mass-dense granules and contractile vacuoles. These results support the idea that mass-dense granules, polyphos-

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1 The abbreviations used are: V-H\(^+\)-ATPase, vacuolar H\(^+\)-ATPase; H\(^+\)-PPase, H\(^+\)-pyrophosphatase; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; AMDP, aminomethylenediphosphonate; H\(_p\)DIDS, 4′,4″-disothiocyanatodihydrostilbene-2,2′-disulfonic acid.
Contractile Vacuole Function in Dictyostelium discoideum

Experimental Procedures

Cell Cultures—D. discoideum (strain AX-3) was obtained from the American Type Culture Collection. Cells were grown axenically in ATCC culture medium 1034/modified PYNH medium (containing 10 g/liter peptone, 10 g/liter yeast extract, 1 g/liter yeast nucleic acid, 15 mg/liter folic acid, 1 mg/liter hemin, 18.1 g/liter KH₂PO₄, 25 g/liter Na₂HPO₄, and 10% fetal bovine serum) at pH 6.5 at room temperature and harvested at the late exponential growth phase.

Chemicals—ATP, Dulbecco’s PBS, antibody against calmodulin, acetoxyazolomide, and reagents for marker enzyme assays were from Sigma. Sodium carbonate (400 mmol/L) solution; OptiPrep, Nycodent were obtained from Invitrogen. Bafilomycin A₁ was from Kamiya Biomedical Co. (Thousand Oaks, CA). Monoclonal antibody N-2 against the 110-kDa accessory protein of the V⁻H⁻ATPase of D. discoideum (31) was obtained from the Monoclonal Antibody Center of the University of Hawaii. Polyonal antibodies raised against a keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to hydrophilic loop IV (antibody PAB₉₆K or 324) of plant V⁻H⁻ATPase (32) and aminomethylphosphonate, NADPH, catalase (33) were kindly provided by Professor Philip A. Rea (University of Pennsylvania, Philadelphia, PA). Affinity-purified polyclonal antibody against the Ca²⁺-ATPase PAT1 (31) was kindly provided by Professor Barrie Coukell (York University, Toronto, Canada). A monoclonal antibody against a keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to hydrophilic loop XII of Trypanosoma cruzi H⁻PPase (34) was prepared at the University of Illinois Biotechnology Center. Molecular mass markers and Coomassie blue protein assay reagent were from Bio-Rad. The EnzChek phosphate assay kit and a rhodamine-coupled goat anti-mouse IgG secondary antibody (1:150), respectively. Control preparations were incubated with preimmune serum or without the primary antibody. Immunofluorescence images were obtained with an Olympus laser scanning confocal microscope using optical sections of 0.1 μm (40). For polyphosphate localization, cells were washed twice with Dulbecco’s PBS and resuspended in the same buffer at a density of 10⁶ cells/ml. 45 μL of this suspension was incubated at room temperature with 10 μg of DAPI. After 10 min, the samples were mounted on a slide and observed with the Olympus laser scanning confocal microscope using optical sections of 0.1 μm and an argon laser for detection of polyphosphate (40).

Electron Microscopy and X-ray Microanalysis—For imaging whole cells and electron-dense fractions, the preparations were washed with 0.25 mm sucrose, and a 5-μL sample was placed on a Formvar-coated copper grid, allowed to adhere for 10 min at room temperature, blotted dry, and observed directly with a Hitachi 600 transmission electron microscope operating at 100 kV (21). Energy-dispersive x-ray analysis was done at the Electron Microscopy Center of Southern Illinois University (Carbondale, IL). Specimen grids were examined in a Hitachi H-7100FA transmission electron microscope at an accelerating voltage of 50 kV. Fine probe sizes were adjusted to cover the electron-dense vacuoles (or a similar area of the background), and x-rays were collected for 100 s by utilizing a thin-window detector (Norvar). Analysis was performed using a Noran Voyager III analyzer with a standardless analysis identification program.

For osmotic shock treatment, cells were washed twice with Dulbecco’s PBS and resuspended in Dulbecco’s PBS diluted 1:10. After 5 min of incubation, samples were placed on Formvar-coated copper grids and observed as described above.

Measurement of the Contractile Vacuole Cycle—Cells (10⁶) were washed twice with Dulbecco’s PBS and resuspended in 50 μL of the same buffer. Then, acetazolamide (0.25, 0.5, and 1 mm), H₂DIDS (250, 400, and 500 μM), bafilomycin A₁ (1, 2, and 5 μM), or AMDP (20 μM) was added, and the cell suspension was placed in a hemocytometer and examined by light microscopy using a Zeiss photomicroscope. The contractile vacuole cycle is defined as the time (in seconds) between two vacuolar discharges on the same cell. Two contractile vacuole cycles per cell of at least 10 cells per condition were measured. Results are expressed as an average of the values obtained in two different incubations from three independent experiments.

Carbonic Anhydrase Activity Measurements—Carbonic anhydrase activity was measured as described (42) with some modifications. Briefly, 30 μL Tris-HCl, pH 7.8, 1 mM p-nitrophenyl acetate, different from the amount of P, released upon treatment with inorganic pyrophosphatase (final activity, 10 units/ml; Sigma) as previously described (40).

Characterization of Pyrophosphatase Activity—Pyrophosphatase was assayed by measuring released phosphate using the EnzChek phosphatase assay kit (35). The reaction mixture contained 10 mM MgCl₂, 10 mM Hepes, pH 7.2, 2 mM MgSO₄, 50 μM EGTA, 0.1 mM 2-amino-6-mercapto-7-methylpyrrole ribonucleoside, 0.4 units/ml purine-nucleoside phosphorylase, 0.2–0.4 mg of electron-dense vacuole fraction, and PP, as indicated in a total volume of 0.1 mL. Activity was recorded at 360 nm and 30 °C in a PowerWave 340i plate reader (Bio-Tek Instruments, Winooski, VT).

Immunoblot Method—Aliquots (15 μL, 20 μg of protein) of the D. discoideum mass-dense granule fraction were mixed with 15 μL of electrophoresis buffer (125 mM Tris-HCl, pH 7, 10% (v/v) b-mercaptoethanol, 20% (v/v) glycerol, and 4% (w/v) bromphenol blue) and boiled for 5 min prior to application to 10% SDS-polyacrylamide gels. Electrophoresed proteins were transferred to nitrocellulose using a Bio-Rad Trans-Blot apparatus. Membranes were blocked in 5% nonfat dry milk in PBS and kept overnight at 4 °C. A 1:10,000 dilution of antibody 324 against H⁻PPase in blocking buffer was applied to blots at room temperature for 60 min. The nitrocellulose was washed three times for 20 min each with PBS (containing 0.1% (v/v) Tween 20) before the addition of a 1:2,000 dilution of goat anti-rabbit IgG in blocking buffer for 60 min. Immunoblots were visualized on radiographic film (Eastman Kodak Co.) using the ECL chemiluminescence detection kit (Amersham Biosciences, Inc.).

Immunofluorescence Microscopy—Cells fixed with formaldehyde (freshly prepared) were allowed to adhere to poly-l-lysine-coated coverslips, permeabilized with 0.3% Triton X-100 and 3% albumin for 5 min, and prepared for immunofluorescence with a 1:1,000 dilution of polyclonal antibody 324 or anti-PAT1 or anti-c alcaldomin antibody and a 1:100 dilution of monoclonal antibody N-2 or a monoclonal antibody against T. cruzi H⁻PPase (41) and a rhodamine-coupled goat anti-rabbit or fluorescein isothiocyanate-coupled goat anti-mouse IgG secondary antibody (1:150), respectively. Control preparations were incubated with preimmune serum or without the primary antibody. Immunofluorescence images were obtained with an Olympus laser scanning confocal microscope using optical sections of 0.1 μm (40). For polyphosphate localization, cells were washed twice with Dulbecco’s PBS and resuspended in the same buffer at a density of 10⁶ cells/ml. 45 μL of this suspension was incubated at room temperature with 10 μg of DAPI. After 10 min, the samples were mounted on a slide and observed with the Olympus laser scanning confocal microscope using optical sections of 0.1 μm and an argon laser for detection of polyphosphate (40).

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Extraction and Analysis of Long- and Short-chain Polyphosphates and PP.—Cells (1 × 10⁶ to 1 × 10⁷) were washed once with Dulbecco’s PBS and treated to extract either long- or short-chain polyphosphate. Different samples were used in each case. Long-chain polyphosphate extraction was performed as described by Ault-Riche et al. (39). For PP, and short-chain polyphosphate extraction, the cell pellet was resuspended in ice-cold 0.5 M HClO₄ (2 mL/g of cells (wet weight)). After 30 min of incubation on ice, the extracts were centrifuged at 14,000 × g for 30 s. The supernatant was neutralized with 0.72 M KOH and 0.6 M KHCO₃. Precipitated KClO₃ was removed by centrifugation at 14,000 × g for 30 s, and the extract supernatant was used for polyphosphate and PP determination. Polyphosphate levels were determined from the amount of P, released upon treatment with an excess of purified recombinant exoprophosphatase (PFX1) from Saccharomyces cerevisiae as previously described (40). PP, levels were determined as a result of a specific assay for PP measured as described (42) with some modifications.
Concentrations of acetazolamide, and 10 μl of the sample were mixed in a final volume of 300 μl. Changes in absorbance at 348 nm and 30 °C were determined in the PowerWave 340i plate reader.

RESULTS

Elemental Analysis and Isolation of Electron-dense Vacuoles of D. discoideum—Mass-dense granules are recognized by their high electron density when they are observed in transmission electron micrographs of cryosectioned cells (13, 14). Abundant vacuoles with high electron density of varying diameter (average of 200 nm) were seen when whole D. discoideum amebas were observed by transmission electron microscopy without fixation and staining (Fig. 1A). X-ray microanalysis was performed on these vacuoles (Fig. 1B). The spectrum shown is the one that yielded the most counts in 100 s (of 10 spectra obtained), but all other spectra taken from mass-dense granules were qualitatively similar: counts for phosphorus were —3-fold greater than counts for calcium, which were about the same as counts for magnesium. Peaks for calcium, phosphorus, and magnesium were not present in spectra taken from the background (Fig. 1C). Peaks for copper arose from the grid.

To purify these mass-dense granules and to investigate their chemical and enzymatic content, we adapted the purification procedure used for the isolation of similar electron-dense vacuoles (acidocalcisomes) from T. cruzi (26). Assaying marker enzymes (Fig. 2) assessed the utility of the method. Pyrophosphatase (a marker of acidocalcisomes) was assessed as the pyrophosphate hydrolytic activity sensitive to the specific H+/PPase inhibitor AMDP (33). Its yield in the polyphosphate body fractions (fractions 1 and 2) was 25%, whereas the yield of protein in the same fractions was only 0.9%, a 125-fold purification of the polyphosphate bodies, as a pyrophosphatase activity was also present in the contractile vacuole bladders of D. discoideum (see below). Mitochondria (marked by succinate-cytochrome c reductase), lysosomes (marked by acid phosphatase), and contractile vacuoles (marked by alkaline phosphodiesterase and alkaline phosphatase) (35) were not enriched in this fraction. The polyphosphate body fractions (fractions 1 and 2) contained ~20% of the total V-H+-ATPase (measured as the 0.5 μM bafilomycin A1-sensitive ATP hydrolytic activity), 36% of the Ca2+-ATPase (measured as the 10 μM calcium-stimulated ATP hydrolytic activity) (Fig. 2), 36% of the total amount of PPi, and >35 and 45% of the total amounts of short- and long-chain polyphosphates, respectively (Fig. 3). Electron microscopy of the mass-dense granule fractions (fractions 1 and 2) by observation of air-dried samples (Fig. 1D) showed the presence of electron-dense granules with the same appearance as the mass-dense granules observed in the preparation of whole cells (Fig. 1A). The results of x-ray microanalysis of the isolated granules (Fig. 1E) were similar to those obtained with whole cells (Fig. 1B), except that proportionally less calcium and magnesium were detected, probably due to ionic changes occurring during the fractionation procedure.

An H+/PPase in the Mass-dense Granule Fraction of D. discoideum—The pyrophosphatase activity detected in the mass-dense granule preparations (Fig. 1) was specific for the H+/PPase activity, as the 10 μM calcium-stimulated ATP hydrolytic activity (Fig. 2) of the mass-dense granule fraction was quantitatively similar to that of the isolated polyphosphate bodies. The pyrophosphatase activity detected in the mass-dense granule fraction (Fig. 1) was specific for the H+/PPase activity, as the 10 μM calcium-stimulated ATP hydrolytic activity (Fig. 2) of the mass-dense granule fraction was quantitatively similar to that of the isolated polyphosphate bodies.
Dense granule fraction of *D. discoideum* (Fig. 2) was measured by inorganic phosphate detection (21) in the presence of different buffers. Control pyrophosphatase activity was 0.16 ± 0.009 µmol of pyrophosphate consumed per min/mg of protein (means ± S.E. of results from five separate experiments) and was inhibited by 30 µM AMDP by 63 ± 8.3% (means ± S.E. from four experiments). The effects of monovalent cations on AMDP-inhibitory pyrophosphate hydrolysis are shown in Table I. Replacing 130 mM KCl with 250 mM sucrose in the buffer resulted in lower pyrophosphate hydrolysis than in the presence of 130 mM KCl or 65 mM NaCl and 125 mM sucrose. These results suggest that K⁺-stimulated this activity, whereas Na⁺ was inhibitory.

The dependence of the initial rate of pyrophosphate hydrolysis on pyrophosphate concentration in the *D. discoideum* mass-dense granule fraction is shown in Fig. 4A. Activity was maximal at ~30 µM pyrophosphate, with an apparent *Kₘ* of 6.3 µM. Fig. 4B shows the effect of medium pH on the initial rate of pyrophosphate hydrolysis in the *D. discoideum* mass-dense granule fraction. Activity was optimal at pH 7.2. Maximal stimulation of pyrophosphatase activity by KCl was obtained at ~30 mM KCl (Fig. 4C).

**Inhibition of H⁺-PPase Activity**—Pyrophosphate hydrolysis of the mass-dense granule fraction was inhibited by the pyrophosphate analogs AMDP and amidophosphoribosylpyrophosphate in a dose-dependent manner (Fig. 5, A and B). Some residual pyrophosphate hydrolytic activity could be detected even at 100 µM AMDP. There may be an AMDP-insensitive pyrophosphatase activity present in the fraction as well as the H⁺-PPase (see also Table I). Potassium fluoride (Fig. 5C) and the thiol reagent N-ethylmaleimide (Fig. 5D), agents known to inhibit the H⁺-PPases from plants (28, 29), trypanosomatids (22, 23, 34), and apicomplexan parasites (27, 43, 44), were also effective in inhibiting the *D. discoideum* pyrophosphatase activity in a dose-dependent manner.

**Immunological Evidence for the Co-localization of the H⁺-PPase with Calmodulin, V-H⁺-ATPase, and Ca²⁺-ATPase**

We investigated the localization of the H⁺-PPase in *D. discoideum* amebas by immunocytochemistry using an antibody against a conserved peptide of Arabidopsis thaliana H⁺-PPase. Antibody 324 showed cross-reactivity with a band of 63 kDa present in the *D. discoideum* pyrophosphatase activity in a dose-dependent manner.

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**Co-localization Studies**

Co-localization studies were also done using antibodies against the H⁺-PPase (Fig. 6E) and a monoclonal antibody that recognizes the 110-kDa accessory protein of the V-H⁺-ATPase (Fig. 6F) (31). Using confocal microscopy, we observed co-localization of the two proton pumps in both the contractile vacuole and smaller cytoplasmic vacuoles (areas in yellow in Fig. 6G). These results are in agreement with the co-localization of the...
V-H+–ATPase and the H+–PPase in the mass-dense granules and contractile vacuoles as assayed biochemically (Fig. 2). Co-localization of the H+–PPase (using a monoclonal antibody against T. cruzi H+–PPase) (Fig. 6F) with the Ca2+–ATPase PAT1 (using a polyclonal antibody against PAT1) (11) (Fig. 6J) in both the contractile vacuole and smaller vacuoles was also detected, as shown by the areas in yellow in Fig. 6K.

Further Evidence for the Localization of Polyphosphate in the Polyphosphate Bodies and Contractile Vacuoles—Because the subcellular fractionation studies suggested the localization of polyphosphate in both the mass-dense granules and contractile vacuoles, we also investigated the location of polyphosphate using DAPI (Fig. 7). DAPI has been shown to shift its emission fluorescence to a maximal wavelength of 525 nm in the presence of polyphosphate, this change being specific for polyphosphate (polyphosphate bodies) apparently concentrated in the region occupied by the contractile vacuole (Fig. 7), we submitted the amebas to osmotic shock to obtain a sudden increase in the size of the contractile vacuole and to allow a better visualization of its interaction with mass-dense granules. Mass-dense granules were found close to the contractile vacuole membrane (Fig. 8, C and D, arrows), in contact with its membrane (A and C, black arrowheads), or inside the vacuole (B and C, white arrowheads).
the interval between two contractile vacuole discharges in the same cell (Fig. 9A). Because an intravacuolar carbonic anhydrase would facilitate the flux of bicarbonate, we tested whether the carbonic anhydrase inhibitor acetazolamide also prolonged the contraction cycle of the contractile vacuoles. As expected, this was the case (Fig. 9B). To confirm that a carbonic anhydrase was present in the contractile vacuole, we measured this activity in the fractions obtained by iodixanol gradient centrifugation (Fig. 2). We found that most of the 1 mM acetazolamide-sensitive carbonic anhydrase activity co-localized with the contractile vacuole markers (compare Fig. 9C with Fig. 2), whereas another, acetazolamide-insensitive activity was preferentially present in other fractions.

**DISCUSSION**

We have demonstrated that vacuolar-type proton-transporting ATPase, Ca\(^{2+}\)-ATPase, and H\(^{+}\)-PPase activities co-localize with PP\(_1\) and polyphosphate in the mass-dense granules purified from *D. discoideum* (Figs. 2 and 3). Therefore, these organelles are very similar to acidocalcisomes isolated from trypanosomatids and apicomplexan parasites (19), and the results extend the range of organisms shown to possess this type of organelle. The potential for purifying this organelle leads to...
It has been proposed that the contractile vacuoles of D. discoideum are typically composed of two forms of membrane: individual, large, empty, excretory vacuoles (bladders) surrounded by extensive accessory membranes (spongiosomes) (8). It was proposed that the spongiosomes collect water from the cytoplasm and deliver it to the bladder for expulsion after its contact with the plasma membrane (8). Using subcellular fractionation techniques, it was possible to separate a buoyant fraction containing the bladders and a slightly denser fraction containing the spongiosomes (8). This latter subcellular fraction was named acidosomes (7) and shown to contain V-H\textsuperscript{+}-ATPase and H\textsuperscript{+}-countertransporting Ca\textsuperscript{2+}-ATPase activities (3). Although one report showed the presence of peg-like particles attributed to the V-H\textsuperscript{+}-ATPase in both bladder and spongiosome membranes (9), another report showed their absence in bladders (8), suggesting either the presence of only the catalytically inactive pump base pieces in bladders or the presence of spongiosome sacules in D. discoideum that could have been taken as bladders by Heuser et al. (9). Our results clearly identify two main subcellular fractions containing V-H\textsuperscript{+}-ATPase and Ca\textsuperscript{2+}-ATPase activities. One is buoyant and contains contractile vacuole enzymatic markers (alkaline phosphatase and alkaline phosphodiesterase), whereas the other is very dense and corresponds to the mass-dense granule fraction. The acidosomal or spongiosome fraction described by some authors (8) may co-sediment with the bladders by our fractionation method given the steep density gradient in the upper part of the iodixanol gradients after centrifugation (Fig. 2). The presence of polyphosphates in the contractile vacuole bladders also argues against the idea that they are empty inside and that they contain only water or a dilute electrolyte (9). As discussed by Heuser et al. (9), the basic problem with this view is that this reservoir would have to possess an exceptionally low water permeability to resist passive water egress as its ions are being reabsorbed and its contents are becoming hypotonic relative to the cytoplasm. This is against results indicating that the bladders have normal water permeability (9). In this regard, an earlier hypothesis suggested that contractile vacuoles might be filled with an expandable hydrocolloid that accumulates and retains water (50). The presence of large amounts of short- and long-chain polyphosphates appears to favor this hypothesis.

The mechanism by which the contractile vacuoles extract water from the cytoplasm has not been completely elucidated (9). It has been suggested that the V-H\textsuperscript{+}-ATPase provides the driving force for filling the vacuoles by transporting protons from the cytoplasm to the lumen, followed by antiport of cytosolic osmotolyte and a passive or osmotic influx of water (8). The presence of an H\textsuperscript{+}-PPase could provide an additional driving force for water uptake. In conclusion, the mass-dense granules of D. discoideum are similar to the acidocalcisomes of trypanosomatid and apicomplexan protozoa (19) and are linked to the contractile vacuole complex of this organism.

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