Acidocalcisomes and the Contractile Vacuole Complex Are Involved in Osmoregulation in *Trypanosoma cruzi*\(^*\)\(^\S\)

Peter Rohloff\(^\S\), Andrea Montalvetti\(^\S\), and Roberto Docampo\(^\S\)

From the Laboratory of Molecular Parasitology, Department of Pathobiology and Center for Zoonoses Research, University of Illinois at Urbana-Champaign, Urbana, Illinois 61802

*Trypanosoma cruzi*, the etiologic agent of Chagas disease, resists extreme fluctuations in osmolarity during its life cycle. *T. cruzi* possesses a robust regulatory volume decrease mechanism that completely reverses cell swelling when submitted to hypo-osmotic stress. The efflux of amino acids and K\(^+\) release could account for only part for this volume reversal. In this work we demonstrate that swelling of acidocalcisomes mediated by an aquaporin and microtubule- and cyclic AMP-mediated fusion of acidocalcisomes to the contractile vacuole complex with translocation of this aquaporin and the resulting water movement are responsible for the volume reversal not accounted for by efflux of osmolytes. Contractile vacuole bladders were isolated by subcellular fractionation in iodixanol gradients, showed a high concentration of basic amino acids and inorganic phosphate, and were able to transport protons in the presence of ATP or pyrophosphate. Taken together, these results strongly support a role for acidocalcisomes and the contractile vacuole complex in osmoregulation and identify a functional role for aquaporin in protozoal osmoregulation.

The obligate intracellular parasite *Trypanosoma cruzi* is the causative agent of Chagas disease, which is the leading cause of cardiac death in endemic areas throughout Latin America. More than 11 million people are infected with the parasite, and some 40 million more are at risk (1).

As *T. cruzi* passes through its digenetic life cycle, it encounters many fluctuations in environmental conditions to which it must adapt in order to survive. Extreme fluctuations in osmolarity occur within the gut of the vector (2, 3), and also when the infective form of the parasite passes out of the vector in the highly concentrated excreta and rapidly encounters the interstitial fluid of the mammalian host with a much lower osmolarity.

Physiological adaptations to hypo-osmotic stress have been studied extensively in a wide range of mammalian cell types as well as in unicellular eukaryotes. Upon exposure to a reduction in external osmolarity, cells initially swell but soon regain nearly normal cell volume by a process that has been termed the regulatory volume decrease (RVD); reviewed in Ref. 4), which is accomplished by the efflux of various inorganic ions (such as Na\(^+\) and K\(^+\)) and organic osmolytes to the extracellular environment. Whereas in vertebrate cells inorganic ion efflux is much more important than organic osmolyte efflux, the most functionally significant efflux in unicellular eukaryotes, in terms of total contribution to RVD, seems to involve amino acids.

Previous studies (5) on the response of *T. cruzi* to hypo-osmotic stress have shown that both insect and vertebrate stages possess a robust RVD mechanism that completely reverses cell swelling following a 50% reduction in extracellular osmolarity. Approximately 50% of this compensatory volume reversal could be accounted for by the efflux of cytosolic amino acids (5). Subsequent studies of other major osmolytes, such as sodium, potassium, chloride, inositol, methylamines, P\(_r\), and pyrophosphate (P\(_{pp}\)), failed to reveal another efflux mechanism that could account for the remaining volume reversal, except for K\(^+\) release that could account for only about 7% of the RVD (6).

In many unicellular eukaryotes the adaptation to hypo-osmotic stress involves, in addition to the release of ions and osmolytes as occurs in mammalian cells (7), the release of water by a contractile vacuole complex (CVC). Recent work (8) has shown that most, if not all, CVCs are composed of a two-compartment system enclosed by two differentiated membranes. One membrane (spongiome), which is often divided into numerous vesicles and tubules, contains a proton-translocating V-H\(^+-\)ATPase that provides an electrochemical gradient of protons for water transport and that can fuse only with the membrane of the second compartment. The membrane of the second compartment (bladder) expands into a reservoir for water storage and is capable of fusing with the plasma membrane. It is this second compartment that periodically undergoes contraction, with the expulsion of water (8). In addition, other vacuoles besides the contractile vacuole bladder have been observed to take up water when protozoa are placed in hypo-osmotic media (9, 10), and they have been suggested also to play a role in volume homeostasis (11).

We have recently found that an aquaporin or a water channel is present in the contractile vacuole complex as well as in the acidocalcisomes of *T. cruzi* and suggested a role for these

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\(\S\) Both authors contributed equally to this work.

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\(\S\) To whom correspondence should be addressed: Laboratory of Molecular Parasitology, Dept. of Pathobiology and Center for Zoonoses Research, College of Veterinary Medicine, University of Illinois at Urbana-Champaign, 2001 S. Lincoln Ave., Urbana, IL 61802. Tel.: 217-333-3845; Fax: 217-244-7421; E-mail: rodoc@uiuc.edu.

1 The abbreviations used are: RVD, regulatory volume decrease; AMPD, aminomethylenediphosphonate; CVC, contractile vacuole complex; V-H\(^+-\)PPase, vacuolar proton pyrophosphatase; P\(_r\), inorganic phosphate; P\(_{pp}\), pyrophosphate; polyP, polyphosphate; DCCD, N,N\(^-\)dicyclohexylcarbodiimide; AM, tetraacetoxymethyl ester; BAPTA, 1,2-bis(oxy)ethane-N,N\(^-\),N\(^-\)-tetraacetic acid; GFP, green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; PBS, phosphate-buffered saline; TcAQP, *T. cruzi* aquaporin.
organelles in osmoregulation (12). Acidocalcisomes are acidic calcium-containing organelles present in a number of unicellular eukaryotes (13–15), as well as in bacteria (16), that have been postulated to be involved in osmoregulation because they change their polyphosphate (polyP) (17) and ionic (18) content when submitted to osmotic changes. It has been postulated that acidocalcisomes could act as a subcellular, osmotically active reservoir linked to the contractile vacuole function in both Chlamydomonas reinhardtii (14) and Dictyostelium discoideum (15). In addition, acidocalcisomes could be the “vesicles” or “vacuoles” that have long ago been identified in free-living protozoa as transferring either water or osmolytes and ions to the spongione linked to the CV (8), because, as acidocalcisomes (13), they usually appear as indistinguishable empty vesicles when examined under conventional transmission electron microscopy (8).

In this study we have examined the role of the acidocalcisomes and the CVC in osmoregulation in T. cruzi. Hypo-osmotic stress resulted in a significant increase in cyclic AMP, swelling of the acidocalcisomes, and displacement of green fluorescent protein (GFP)-T. cruzi epimastigotic aquaporin (Tc-AQP) immunofluorescence labeling from the acidocalcisomes to the CVC in a microtubule- and cyclic AMP-dependent fashion. In addition, we isolated the contractile vacuole bladders, showed that they are enriched in alkaline phosphatase, V-H+-ATPase, and calmodulin, and found that they have large amounts of basic amino acids and P, and that they are able to transport protons in the presence of ATP or PPi, RVD was inhibited by aquaporin inhibitors, by an intracellular Ca2+ chelator, and by a vacuolar H+-ATPase proteolipid subunit inhibitor, suggesting fusion of acidocalcisomes with the CVC. These fusion events with the CVC were also recorded. These results support a role for acidocalcisomes and the contractile vacuole complex in osmoregulation and identify a functional role for aquaporin in protozoal osmoregulation.

EXPERIMENTAL PROCEDURES

Cell Culture—T. cruzi epimastigotes (Y strain) were grown at 28 °C in liver infusion tryptose medium (19) supplemented with 10% heat-inactivated fetal bovine serum and 1% newborn calf serum. GFP-T. cruzi epimastigotic aquaporin (GFP-Tc-AQP)immunofluorescence labeling was performed on cells that were grown at 28 °C in liver infusion tryptose medium supplemented with 10% heat-inactivated fetal bovine serum and 1 mg/ml geneticin.

Chemicals—Mammalian protease inhibitor mixture, β-glycerophosphate, dipirydiamole, 3-isobutyl-1-methylxanthine (IBMX), 2-deoxy-3-AMP, and cycloheximide were from Sigma. GPP-N9262, OAQ-mono- and OAQ-di-epimastigotes (12) were maintained in liver infusion tryptose medium supplemented with 10% heat-inactivated newborn calf serum and 1 mg/ml geneticin.

Cell Volume Measurements—Relative changes in cell volume after induction of hypo-osmotic stress were followed by using a light scattering technique as described previously (5, 6).

Cyclic AMP and Cyclic GMP Determinations—Cells were suspended at a concentration of 2 × 106 per ml and preincubated with 1 mM IBMX for 10 min in Dulbecco’s PBS supplemented with 5 mM glucose. Then they were diluted with either water or buffer to 1 × 106 cells per ml. Aliquots of 0.5 ml were taken at the times indicated, centrifuged, and resuspended in 100 μl of PBS and 400 μl of 50 mM sodium acetate, pH 5.5, preheated to 95 °C. These samples were heated at 95 °C for 5 min, centrifuged, and the supernatants collected and stored at −20 °C. Cyclic AMP or cyclic GMP measurements were done using the Amersham Biosciences kit with the acetylation protocol.

Fluorescence Microscopy—For conventional electron microscopy, cells or subcellular fractions were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde, washed in Dulbecco’s PBS, and then embedded in epoxy resin, sectioned, and stained using standard methods. Grids were observed using an Hitachi 600 transmission electron microscopy operating at 75 kV. For morphometric analysis of acidocalcisomes, the diameter of acidocalcisomes in randomly selected cells was measured at 100× magnification. At least 40 acidocalcisomes were measured from each treatment group (hypo-osmotic, isosmotic) in each of four independent experiments.

Fluorescence Microscopy and Immunofluorescence—For immunofluorescence, cells were fixed with 4% paraformaldehyde, adhered to poly-L-lysine coverslips, permeabilized for 5 min with Dulbecco’s phosphate-buffered saline (PBS), 0.3% Triton X-100, blocked for 1 h with PBS, 3% bovine serum albumin, 1% fish gelatin, 5% goat serum, 50 mM NH4Cl, and incubated with primary antibody for 1 h and then secondary antibody for 45 min. For visualization of the vacuolar H+-ATPase, the SB1 monoclonal antibody (Molecular Probes) was used at 1:10, followed by goat anti-mouse Alexa 546 nm conjugate at 1:1000. For detection of calmodulin, goat polyclonal anti-calmodulin antibody (Santa Cruz Bio-technology) was used at 1:50, followed by rabbit anti-goat Alexa 546 conjugate at 1:1000. Specimens were observed with a Leica TCS SP2 laser-scanning confocal microscope.

For assessment of GFP-Tc-AQP translocation, cells were exposed to hypo-osmotic stress for various times, fixed with 4% paraformaldehyde, adhered to poly-L-lysine coverslips, and visualized by epifluorescence microscopy. For each treatment or time point, at least 50 cells were randomly selected and examined. A “nontranslocation” phenotype was assigned when all the GFP-Tc-AQP immunofluorescence labeling of GFP-Tc-AQP was observed with the Leica TCS SP2 laser-scanning confocal microscope.

Trypanosome Immobilization and Acidocalcisome Tracking—Epimastigotes expressing GFP-Tc-AQP were immobilized on glass surfaces coated with coral tree lectin (Erythrina cristagalli), which interacts with glycoproteins located on the parasite surface membrane containing oligosaccharides with galactosyl (β-1,4)-N-acetylglucosamine. Cells were immobilized on coverslips in 4- or 8-well plates. The lectin was dissolved in Dulbecco’s PBS at a concentration of 0.5 mg/ml. Glass surfaces coated with poly-L-lysine were treated with the lectin solution for 30 min. The solution was then removed, and the lectin-coated surface allowed to air-dry under a laminar flow hood. Epimastigotes in logarithmic phase of growth were collected by centrifugation, washed three times with PBS, and resuspended in buffer A without glucose (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 50 mM Hepes, pH 7.4). The osmolality of the buffer was adjusted to 300 ± 5 mOsm as verified by an Advanced Instruments 3DS Osmometer (Norwood, MA). A 50% hypo-osmotic stress was induced by 1:1 dilution of cell suspensions in buffer A with deionized water.

Video Microscopy—For microscopic analysis of contractile vacuole filling and vesicle fusion, epimastigotes were immobilized on glass slides with coral tree lectin as described above, bathed in 150 mOsm hypo-osmotic buffer, and viewed with a Zeiss Axiovert 100 inverted microscope. Time lapse photographic data were collected at 1-s intervals using a Roper Scientific CoolSnap camera and MCID software. Video sequences were reconstructed using QuickTime and Final Cut Pro software.
Cytchemistry—For cytochemical detection of alkaline phosphatase, a modification of a method described previously was used (22). Cells were fixed in 2.5% glutaraldehyde, 50 mM cacodylate, pH 6.8, 200 mM sucrose, washed twice for 30 min in the same buffer, and then resuspended in 0.5 ml of reaction medium containing 50 mM Tris-HCl, pH 9.0, 200 mM sucrose, 20 mM β-glycerophosphate, and 2.6 mM lead citrate. Lead citrate was prepared as described previously (23). The cell suspension was frozen in liquid nitrogen for 3 min to permeabilize the cells and then thawed by the addition of 4.5 ml of reaction medium. The suspension was frozen at room temperature for 30 min, washed three times with 50 mM cacodylate, pH 6.8, 200 mM sucrose, and embedded in epoxy resin for visualization by transmission electron microscopy.

Western Blotting—Proteins were resolved by SDS-PAGE and blotted onto nitrocellulose membranes using standard methods. All the following steps were performed in Dulbecco's PBS containing 0.1% Tween 20. Membranes were blocked overnight in 5% nonfat dry milk and washed three times, incubated with polyclonal anti-aquaporin antibody at 1:1000 for 1 h and washed three times, incubated with goat anti-rabbit horseradish peroxidase at 1:10,000 for 1 h and washed three times, incubated with horseradish peroxidase at 1:10,000 for 1 h and washed three times, and then the bands were detected by chemiluminescence.

Contractile Vacuole Isolation and Characterization—Contractile vacuoles and acidocalcisomes were isolated simultaneously on discontinuous iodixanol density gradients by using a modification of a method described previously (24). A cell pellet collected from 100 ml of late-log epimastigote culture was disrupted by grinding with silicon carbide; the lysate was clarified by three low speed centrifugations (two times at 36 × g and one time at 144 × g), and the resulting supernatant was centrifuged at 100,000 × g for 90 min. The resulting pellet was loaded into the 24% layer of an iodixanol gradient containing 4-ml steps of 15, 20, 24, 28, 34, 37, and 40%. The gradient was centrifuged at 50,000 × g for 60 min, and 1-ml fractions (27 in all) were collected from the top.

Aliquots of each collected fraction were assayed for enzymatic markers of lysosomes (α-mannosidase), acidocalcisomes (vacular-type (AMDP-sensitive) H⁺-pyrophosphatase), mitochondria (alanine aminotransferase), glycosomes (hexokinase), and contractile vacuole (alkaline phosphatase) as described previously (14, 15, 25). Vacular-type (bafilomycin A₁-sensitive) H⁺-ATPase was assayed as described previously (14). Aliquots were also assayed for amino acid, P₃, and polyP content as described previously (5, 17).

Acidification of isolated contractile vacuole fractions was assayed by measuring changes in the absorbance of acridine orange (A₄₉₀-A₅₃₀ nm) as described previously (26) in an SLM-Aminco DW2000 dual wavelength spectrophotometer. Aliquots of contractile vacuole fractions were incubated at 30 °C in 2.5 ml of 65 mM KCl, 125 mM sucrose, 2 mM MgSO₄, 10 mM K₂HPO₄, 50 mM EGTA, pH 7.2, containing 3 μM acridine orange. Other compounds (PPi, ATP, NH₄Cl, AMDP, and nigericin) were added at the times and concentrations indicated in the appropriate figure legend.

Membrane Integrity and Amino Acid Release—Membrane integrity after treatment with AgNO₃ and HgCl₂ was determined by ethidium bromide exclusion as described before (5). Total amino acid content and amino acid analysis of the supernatants of cells exposed to hypotonic or isotonic buffer were determined as described before (5).

RESULTS

HgCl₂ and AgNO₃ Inhibit the RVD in Epimastigotes—In a previous study (12) we identified an aquaporin (TcAQP) that is located in acidocalcisomes and the contractile vacuole complex of T. cruzi epimastigotes. In contrast to other aquaporins that are inhibited by high micromolar concentrations of either HgCl₂ or AgNO₃ (27, 28), TcAQP was shown to be exquisitely sensitive to inhibition by very low concentrations of these compounds (12). We therefore treated cells with 1 μM of either HgCl₂ or AgNO₃, a concentration shown previously to inhibit osmotic swelling in Xenopus laevis oocytes transfected with TcAQP (12), and we then monitored the rate of volume recovery during hypo-osmotic stress. Relative volume was followed over time using the light-scattering technique.

![Image](http://www.jbc.org/Downloaded_from http://www.jbc.org/Downloaded_from)
as described previously (5), in which changes in absorbance of a cell suspension are negatively correlated with changes in cell volume. Both inhibitors significantly reduced the rate of volume recovery (Fig. 1, A and B). Furthermore, this inhibitory effect was not because of interference with hypo-osmotically induced amino acid efflux (Fig. 1C) and was not because of a general toxic effect of the compounds, because the magnitude of amino acid release was unaffected by pretreatment with either HgCl₂ or AgNO₃ (Fig. 1C). Epimastigotes under isosmotic conditions treated with either 1 μM HgCl₂ or 1 μM AgNO₃ for 5 min showed neither morphological nor motility alterations and did not increase their permeability to ethidium bromide (Fig. 1D). Taken together, these results suggest a central role for TcAQP in the response of the parasites to hypo-osmotic stress. GFP-TcAQP Translocation during Hypo-osmotic Stress—Based on analogy to vasopressin-stimulated translocation of AQP-2-containing vesicles in the mammalian kidney collecting ducts (29, 30), the possibility of a hypo-osmotically induced
Tc:AQP translocation event was studied in GFP-Tc:AQP expressing epimastigotes. As reported before (12), GFP-Tc:AQP localized to acidocalcisomes (arrowheads) and to the contractile vacuole (Fig. 2B, arrow). When cells were stressed and observed microscopically, the GFP-Tc:AQP-containing acidocalcisomes that were closer to the contractile vacuole could be observed migrating toward it (see below). In many cells (Fig. 2D) only the fluorescent spot corresponding to the contractile vacuole could be observed because it became brighter after hypo-osmotic stress, and less fading of this signal than that of the acidocalcisomes occurred. We therefore investigated whether it was possible to image the movement of acidocalcisomes in live cells submitted to hypo-osmotic stress or in the presence of dibutyryl cyclic AMP (500 μM, see below) either by video fluorescence microscopy or by a new method for three-dimensional particle tracking termed scanning fluorescence correlation spectroscopy (21). Because epimastigotes are very motile cells, they needed to be immobilized to follow these changes. This was successfully achieved by attaching them to coverslips covered with coral tree lectin (see “Experimental Procedures”). Periodic filling of the contractile vacuole could be followed by phase microscopy when epimastigotes were incubated in the buffer at 150 mOsm (Fig. 3A; see also movie 1 under Supplemental Material). However, attempts to follow the movement of acidocalcisomes using video fluorescence microscopy, or scanning fluorescence correlation spectroscopy using a two-photon microscope, were unsuccessful, due in part to the small size of the acidocalcisomes and also the fast fading of the GFP signal.

As an alternative method, we exposed cells to hypo-osmotic stress for various lengths of time, fixed them, and counted the percentage of cells exhibiting only one bright spot corresponding to the contractile vacuole. Although this eliminated from consideration the cells that showed labeling of some acidocalcisomes at the end of each incubation period, thus causing an underestimation of the translocation phenotype, it provided a more objective estimate of the changes that occurred. A certain percentage of cells maintained in isosmotic conditions already contained only one bright spot corresponding to the contractile vacuole. However, the percentage of cells containing only one bright spot increased significantly over 5 min of hypo-osmotic stress (Fig. 2, E and F). Taken together, the results are consistent with hypo-osmotically induced translocation of GFP-Tc:AQP toward the contractile vacuole.

Cyclic AMP and Microtubule Dependence of Hypo-osmotically induced Acidocalcisomal Translocation—In analogy to the prominent cyclic AMP dependence of AQP trafficking in several mammalian systems (30–33), the role of this signaling pathway in acidocalcisomal translocation was next investigated. Incubation of cells for 10 min with 250 μM of the membrane-permeable cyclic AMP analog dibutyryl cyclic AMP significantly mimicked hypo-osmotically induced translocation, increasing the percentage translocation close to the same levels as those induced by hypo-osmotic stress (Fig. 2E). Incubation in
isomotic medium with the phosphodiesterase inhibitors dipyridamole (100 μM) or 3-isobutyl-1-methylxanthine (1 mM) also increased the percentage translocation in a significant manner, albeit to levels less than those produced by dibutyryl cyclic AMP (Fig. 2E). Finally, preincubation for 10 min with the adenyl cyclase inhibitor 2-deoxy-3-AMP (100 μM) followed by induction of hypo-osmotic stress significantly decreased the percentage translocation (Fig. 2E).

We then measured intracellular cyclic AMP levels during hypo-osmotic stress, after preincubation of the cells in the presence of a phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine). Epimastigotes were hypo-osmotically stressed, and at appropriate time points, aliquots were processed for cyclic AMP measurement. Resting cyclic AMP levels were in the range of 7.5–15 pmol/1 × 10^9 cells, and maximal increase occurred at 1 min following the induction of hypo-osmotic stress (Fig. 2G). No cyclic AMP accumulation was observed in the absence of the phosphodiesterase inhibitor. Similar experiments were performed for cyclic GMP because this compound has been implicated in the activation of mammalian AQP-1 (34). The cyclic GMP levels detected were somewhat higher than for cyclic AMP (25–35 pmol/1 × 10^9 cells), but no increase was observed following the induction of hypo-osmotic stress (data not shown) either in cells treated or not with phosphodiesterase inhibitors.

Because vesicular transport in most cell types is microtubule-dependent, and cyclic AMP signaling influences microtubule assembly in many well characterized systems (35–37), the effect of microtubule inhibitors on GFP-TcAQP translocation was assessed. Trypanosomatids are resistant to the effects of well known microtubule inhibitors such as colchicine and the benzimidazoles (38). We therefore tested the herbicides trifuralin and chloralin that have been shown to be potent microtubule inhibitors in trypanosomatids (38). Preincubation of the cells for 10 min with chloralin (10 μM) or trifuralin (500 μM) before induction of hypo-osmotic shock significantly inhibited hypo-osmotically induced translocation (Fig. 2F).

Functional Significance of Cyclic AMP Modulators and Microtubule Inhibitors for the RVD—To assess the functional significance of cyclic AMP-modulating agents and microtubule inhibitors, epimastigotes were preincubated for 10 min with various concentrations of these drugs, and then the rate of volume recovery during hypo-osmotic stress was followed by light scattering. Although dibutyryl cAMP did not stimulate volume recovery at 250 or 500 μM, it significantly increased volume recovery at 1 mM (Fig. 4A). Dipyridamole (100–500 μM), 3-isobutyl-1-methylxanthine (1 mM), and 2-deoxy-3-AMP (100 μM to 1 mM) had no effect on volume recovery (data not shown). Chloralin (10 μM) and trifuralin (500 μM) significantly inhibited volume recovery (Fig. 4, B and C) at concentrations similar to those that inhibit GFP-TcAQP translocation (Fig. 2F).

Vacuole Fusion Occurs and Inhibitors of Vacuole Fusion Inhibit RVD—The results described above are compatible with translocation of GFP-TcAQP from the acidocalcisomes to the contractile vacuole and为进一步说明，we propose a model (see also supplemental movie 2). If this were the case, inhibition of membrane fusion would inhibit RVD. All eukaryotic cells use a program of membrane fusion and fission to assemble membranes, both internally and on their surface (39). It has been shown in yeasts that cytosolic Ca^{2+} is required for many membrane fusion processes (40) and that vacuole fusion depends on the DCCD-reactive proteolipid subunit of the V-H^{+}-ATPase (41). In agreement with these requirements, loading of epimastigotes with BAPTA-AM, an intracellular Ca^{2+} chelator, but not with N-[(2-metoxyphenyl)limidaoctic acid-AM, a compound of similar structure to BAPTA-AM but with no Ca^{2+} chelating activity (42), inhibited the RVD (Fig. 5A). The concentration of BAPTA-AM used in these experiments has been shown before to chelate intracellular Ca^{2+} in T. cruzi (5). In addition, preincubation of the cells with DCCD (50 μM) also inhibited RVD (Fig. 5B). Neither chelation of intracellular Ca^{2+} with BAPTA-AM (Fig. 5C, BAPTA) nor preincubation with DCCD (Fig. 5D, DCCD) resulted in inhibition of amino acid release from epimastigotes.

Acidocalcisome Swelling during RVD—Because swelling of acidocalcisomes could also contribute to an increase in cytosolic osmolarity during volume recovery, we investigated whether this phenomenon took place during hypo-osmotic stress. Epimastigotes were fixed 5 min after exposure to hypo-osmotic stress, sectioned, and examined by transmission electron microscopy. The average diameter of acidocalcisomes was determined in randomly selected cells and compared with isosmotic control cells in four experiments. Acidocalcisomes from hypo-osmotically

![Figure 4](http://www.jbc.org/Downloaded_from)
stressed cells has an average diameter of 286 ± 28 nm corresponding to a volume of 12.6 ± 3.8 zl if they are considered perfect spheres. Acidocalcisomes for isosmotic control cells had an average diameter of 252 ± 18 nm corresponding to a volume of 8.5 ± 1.9 zl. These values are statistically different from each other and corresponded roughly to an acidocalcisome volume.
increase of 50% during hypo-osmotic stress.

In Situ Analysis of Contractile Vacuole Markers—To isolate the contractile vacuole of *T. cruzi* and investigate its relationship with acidocalcisomes, we investigated potential markers of these organelles. Several enzymatic markers for the contractile vacuole have been identified in other organisms, including a vacuolar-type H\(^+\)/H\(^+\)-ATPase (43–47), calmodulin (48), and alkaline phosphatase (22, 49, 50). We took advantage of the fact that, in our transfected epimastigote line, GFP-

FIG. 7. Alkaline phosphatase localizes to the contractile vacuole, spongiome, and flagellar pocket. Alkaline phosphatase cytochemistry and transmission electron microscopy analysis were performed on epimastigotes. In addition to intense localization in the flagellar pocket (FP) (A and B), alkaline phosphatase was also detected in the contractile vacuole (CV) (A and C) and spongiome (A and B, tubules outlined with open arrowheads). K, kinetoplast. Scale bars = 0.25 \(\mu\)m.

was noted in tubular structures located around the contractile vacuole and flagellar pocket, which presumably correspond to the spongiome (Fig. 7, A and B, outlined by open arrowheads) and in the region between closely opposed contractile vacuoles and the flagellar pocket (Fig. 7, B and C, arrows).

Isolation and Characterization of the Contractile Vacuole—To complete our analysis of acidocalcisomal coupling to the contractile vacuole, we sought to isolate contractile vacuole bladders and assess the presence of various acidocalcisomal markers, such as inorganic phosphate and polyphosphate, basic amino acids, and H\(^+\)-pyrophosphatase activity. In *D. discoideum*, the contractile vacuole is a very light structure that sediments at the top of an iodixanol gradient designed to isolate acidocalcisomes (15). Therefore, the standard acidocalcisomal isolation protocol (24) was modified to include an additional recovery step designed to maximize the yield of light structures (100,000 \(\times\) g centrifugation) and additional low density iodixanol steps to improve resolution at the top of the gradient.

Alkaline phosphatase has been used as a marker for contractile vacuole isolation in *D. discoideum* (50, 51), and the cytochemical data of Fig. 6 suggested that it might be a useful marker also in *T. cruzi*. The lightest fraction resulting from the subcellular fractionation was enriched in alkaline phosphatase, and so this fraction was presumptively labeled “contractile vacuole” (Fig. 8A, fraction 1). The dense acidocalcisomal fraction, enriched in polyP and AMDP-sensitive pyrophosphatase, sedimented to the bottom of the gradient (fraction 27). The contractile vacuole fraction was well resolved from or-
FIG. 8. Distribution of organelle markers and P<sub>i</sub>, polyP, and amino acid content of iodixanol gradients. A, various enzymatic markers were assayed in 1-ml fractions (1 = top of gradient; 27 = bottom of gradient) and expressed as % of total activity detected. P<sub>i</sub>, polyP (expressed as P<sub>i</sub> units), and amino acids are expressed as µM detected. Results are expressed as mean ± S.E. from at least two independent fractionations. B, aliquots of the same gradient fractions were resolved by SDS-PAGE and probed with anti-aquaporin antibody. The resulting band migrated around 26 kDa.
ganelle markers for mitochondria (alanine aminotransferase), and glycosomes (hexokinase). Lysosomes (H9251-mannosidase) were not appreciably concentrated in any fraction of the gradient. The presumptive contractile vacuole fraction was not highly enriched in the acidocalcisomal markers AMDP-sensitive pyrophosphatase or polyP. However, it was enriched in Pi and total amino acids. Strikingly, amino acid analysis of the contractile vacuole fraction revealed detectable quantities of only four amino acids, valine, lysine, arginine, and ornithine. In other words, the fraction was highly enriched for basic amino acids similarly to the acidocalcisomes (5). The fraction was only slightly enriched in bafilomycin A1-sensitive V-H\textsubscript{11001}-ATPase. Western blot analysis of the gradient with anti-T. cruzi aquaporin antibody showed enrichment in the contractile vacuole and acidocalcisome fractions (Fig. 8B), consistent with the immunofluorescence and immunocytochemical data (12). Strong reaction to the antibody was also seen in fractions 4–15. It is not possible to assign a location for this latter activity, because most organelles peaked around this point, but it may represent ghosts of cells containing intracellular membranes, as we have reported before using similar gradients (26).

The putative contractile vacuole fraction was fixed and analyzed by standard transmission electron microscopy. The fraction was considerably enriched in round or crescent vacuoles (Fig. 9) whose morphology closely resembled those of similar contractile vacuole isolations from D. discoideum (50). As in D. discoideum, many presented with multivesicular or multilamellar elements (Fig. 9, closed arrowheads). In one remarkable preparation, an apparent contractile vacuole bladder was observed with intact spongiome tubule (Fig. 9A, open arrowheads) and a fusing acidocalcisome (Fig. 9A, Ac).

V-H\textsubscript{11545}-pyrophosphatase assay in isolated contractile vacuole fractions. Following the addition of 3 μM acridine orange, vacuolar acidification was followed by dual wavelength spectrophotometry. At various time points 0.1 mM PP\textsubscript{i}, 1 mM ATP, 1 μM nigericin, 20 μM AMDP, or 5 mM NH\textsubscript{4}Cl was added. Traces a–c are representative of those obtained from at least three independent experiments.
DISCUSSION

We reported previously (5, 6) that amino acid and K+ release could account for only 57% of the RVD of epimastigotes of T. cruzi after hypo-osmotic stress and that another mechanism must be involved in this process. We provide evidence here that water movement mediated by a TcAQP is responsible for the remaining fraction of volume recovery.

Concentrations (1 μM) of HgCl₂ and AgNO₃, which are able to inhibit TcAQP when expressed in Xenopus oocytes (12), inhibited the rate of RVD during hypo-osmotic stress (Fig. 1, A and B) without affecting amino acid release (Fig. 1C) and without toxic side effects (Fig. 1D and not shown). Acidocalcisomes, which have TcAQP (12), increased their volume by 50%, and those close to the contractile vacuole trafficked toward it, as revealed by direct observation of cells expressing GFP-TcAQP (Fig. 2). This traffic was stimulated by cyclic AMP analogs or phosphodiesterase inhibitors (Fig. 2E) and was inhibited by adenyl cyclase and microtubule inhibitors (Fig. 2, E and F).

In agreement with this hypothesis, cyclic AMP levels significantly increased 1 min after hypo-osmotic stress (Fig. 2G). Fusion of acidocalcisomes to the contractile vacuole complex was suggested by video microscopy (Fig. 3A) and by electron microscopic observation of the presence of similar electron-dense material in both organelles and their apparent continuity in intact cells (12) or subcellular fractions (Fig. 9A), by the increase in brightness of the contractile vacuole complex after hypo-osmotic stress (Fig. 2D), and by inhibition of the RVD by an intracellular Ca²⁺ chelator and an inhibitor of the vacuolar H⁺-ATPase proteolipid subunit (Fig. 5, A and B), which did not have any effect on amino acid release (Fig. 5, C and D). Both Ca²⁺ and the proteolipid subunit of the V-H⁺-ATPase have been involved in fusion events in eukaryotic cells (40, 41). In agreement with this hypothesis, a cyclic AMP analog stimulated while microtubule inhibitors inhibited the RVD (Fig. 4).

Isolation of the contractile vacuole bladders revealed their enrichment in basic amino acids, previously shown to be virtually concentrated in acidocalcisomes (5), and P₁, the hydrolysis product of polyP (Fig. 8). Because hydrolysis of polyP occurs during hypo-osmotic stress (17) and neither Pi nor hydrolysis product of polyP (Fig. 8). Because hydrolysis of polyP, which have TcAQP (12), increased their volume by 50%, and those close to the contractile vacuole trafficked toward it, as revealed by direct observation of cells expressing GFP-TcAQP (Fig. 2). This traffic was stimulated by cyclic AMP analogs or phosphodiesterase inhibitors (Fig. 2E) and was inhibited by adenyl cyclase and microtubule inhibitors (Fig. 2, E and F).

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Most interestingly, although very high concentrations of dibutyryl cAMP stimulated volume recovery (Fig. 4), none of the other cyclic AMP modulators had any effect on the rate of volume recovery. It is possible that acidocalcisomol translocation involves several separate regulatory systems or steps and that cyclic AMP effects only one of these. Additionally, because there is a high basal level of translocation (see untreated, isosmotic control cells in Fig. 2, E and F), acidocalcisomal translocation may be a constitutive event that is only modulated by cyclic AMP signaling; consequently, very high levels of cyclic AMP-modulating agents (e.g. 1 mM dibutyryl cyclic AMP) are required to see an effect on the rate of volume recovery. Additionally, although the microtubule inhibitors trifluridine and chloralin do not produce any more inhibition of translocation than the adenylate cyclase inhibitor 2-deoxy-3-AMP (Fig. 2, E and F), the former inhibits volume recovery whereas the latter does not. This also suggests steps other than translocation alone in the contractile vacuole dynamics.

At present little is known about the mechanisms of activation of trypanosomatid adenyl cyclases or their role in cell signaling. T. cruzi adenyl cyclases are encoded by a large polymorphic gene family (52). These enzymes conform to the single transmembrane receptor type found in other trypanosomatids and not to the typical 12-transmembrane spanning structure of G-protein-coupled adenyl cyclases. In addition, the T. cruzi catalytic domain is constitutively active in the absence of eukaryotic regulatory factors (52). This is the first report in which an increase in cyclic AMP is correlated with a change in T. cruzi environment and may suggest the activation of either a mechanosensitive adenyl cyclase like the one that occurs in coronary vascular smooth cells (53) or of a mechano-sensitive channel (54) that could lead to the influx of ions, such as Ca²⁺, and activation of the adenyl cyclase upon hypo-osmotic stress. This would be in agreement with previous results (5) showing that cell swelling of T. cruzi is associated with an influx of Ca²⁺ across the plasma membrane that is blocked by inhibitors of L-type voltage-gated Ca²⁺ channels.

Although the contractile vacuole fraction was not enriched in AMDP-sensitive pyrophosphatase (Fig. 8A), this enzyme is present in many other compartments within the cell (55), and it only serves as a marker for the acidocalcisome because of its exceptionally high density in this location (24). Because the results in Fig. 8A are expressed as percentage of total enzymatic activity in the gradient, this lack of enrichment in the contractile vacuole may be due to dilution by simultaneous enrichment in many other compartments. Indeed, immunocytochemical (12) and well as biochemical (Fig. 10) evidence is consistent with the presence of V-H⁺-pyrophosphatase in the contractile vacuole. The lack of enrichment of the gradient in bafilomycin A₁-sensitive ATPase activity in the acidocalcisome is consistent with previous observations (24) and may reflect the loss of catalytic activity during isolation due to dissociation of peripheral subunits (24) or inactivation (56, 57). On the other hand, biochemical (Fig. 10) and immunological (Fig. 6) evidence of its presence in the contractile vacuoles was obtained.

In summary, it is likely that the remaining percentage of the RVD not accounted for by osmolyte efflux can be accounted for by water and osmolyte accumulation intracellularly in acidocalcisomes and subsequent water ejection through the contractile vacuole into the flagellar pocket. This is the first functional demonstration in any protozoan that aquaporin is central to osmoregulation and contractile vacuole functioning. The presence of aquaporin and the fact that the contractile vacuole is also prominent in mammalian stages of the parasite (5) suggests some new potential chemotherapeutic targets.

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REFERENCES

1. Urbina, J. A., and Docampo, R. (2003) Trends Parasitol. 19, 495–501
2. Kellien, A. H., Grospietsch, T., Kieffmann, T., Zerbst-Borofka, I., and Schaub, G. A. (2001) J. Infect. Physiol. 47, 739–747
3. Kellien, A. H., and Schaub, G. A. (2000) Parasitol. Today 16, 381–387
4. Lang, F., Busch, G. L., Ritter, M., Volkli, H., Waldegger, S., Gulbins, E., and Haussinger, D. (1998) Physiol. Rev. 78, 247–306
5. Rohloff, P., Rodrigues, C. O., and Docampo, R. (2000) Mol. Biochem. Parasitol. 126, 219–220
6. Rohloff, P. (2003) Osmoregulation in Trypanosoma cruzi: The Role of the Acidocalcisome. Ph.D. thesis, University of Illinois at Urbana-Champaign
7. Steckel, T. L., Chiarraviglio, L., and Meredith, S. (1997) J. Eukaryotic Microbiol. 44, 503–510
8. Allen, R. D., and Naitoh, Y. (2002) Int. Rev. Cytol. 215, 351–394
9. Cronenwett, D. L., Neuman, J., Walker, F., and Pierce, S. K. (1991) J. Protozool. 38, 565–573
10. Temesvari, L. A., Rodriguez-Paris, J. M., Bush, J. M., Zhang, L., and Cardelli, J. A. (1996) J. Cell Sci. 109, 1479–1485
11. Van Rossum, G. D. V., Russo, M. A., and Schisselbauer, J. C. (1987) Curr. Top. Membr. Transp. 30, 45–74
12. Montalvetti, A., Rohloff, P., and Docampo, R. (2004) J. Biol. Chem. 279, 38673–38682
13. Docampo, R., and Moreno, S. N. (2001) *Mol. Biochem. Parasitol.* **114**, 151–159
14. Ruiz, F. A., Marchesini, N., Seufferheld, M., Govindjee, and Docampo, R. (2001) *J. Biol. Chem.* **276**, 46196–46203
15. Marchesini, N., Ruiz, F. A., Vieira, M., and Docampo, R. (2002) *J. Biol. Chem.* **277**, 8146–8153
16. Seufferheld, M., Vieira, M. C., Ruiz, F. A., Rodrigues, C. O., Moreno, S. N. J., and Docampo, R. (2003) *J. Biol. Chem.* **278**, 29971–29978
17. Ruiz, F. A., Rodrigues, C. O., and Docampo, R. (2001) *J. Biol. Chem.* **276**, 26114–26121
18. LeFurgey, A., Ingram, P., and Blum, J. J. (2001) *Comp. Biochem. Physiol. A* **128**, 385–394
19. Bone, G., and Steinert, M. (1969) *Nature* **178**, 308–309
20. Luo, S., Vieira, M. A., Zhong, L., Graves, J., and Moreno, S. N. J. (2001) *EMBO J.* **20**, 55–64
21. Levi, V., Ruan, Q., Kis-Petikova, K., and Gratton, E. (2003) *Biochem. Soc. Trans.* **31**, 997–1000
22. Quiviger, B., de Chastellier, C., and Ryter, A. (1978) *J. Ultrastruct. Res.* **62**, 228–236
23. Reynolds, D. (1963) *J. Cell Biol.* **17**, 208–212
24. Scott, D. A., and Docampo, R. (2000) *J. Biol. Chem.* **275**, 24215–24221
25. Scott, D. A., Docampo, R., Dvorak, J. A., Shi, S., and Leapman, R. D. (1997) *J. Biol. Chem.* **272**, 28020–28029
26. Scott, D. A., de Souza, W., Benchimol, M., Zhong, L., Lu, H. G., Moreno, S. N., and Docampo, R. (1998) *J. Biol. Chem.* **273**, 22151–22158
27. Hansen, M., Kun, J. F., Schultz, J. E., and Beitz, E. (2002) *J. Biol. Chem.* **277**, 4874–4882
28. Niemietz, C. M., and Tyerman, S. D. (2002) *FEBS Lett.* **531**, 443–447
29. Nielsen, S., Chou, C. L., Marples, D., Christensen, E. I., Kishore, B. K., and Knepper, M. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 1013–1017
30. Gustafson, C. E., Levine, S., Katsura, T., McLaughlin, M., Alexio, M. D., Tamraparni, B. K., Verkman, A. S., and Brown, D. (1998) *Histochem. Cell Biol.* **110**, 377–386
31. Brown, D., Katsura, T., and Gustafson, C. E. (1998) *Am. J. Physiol.* **275**, F328–F331
32. Garcia, F., Kierbel, A., Larocca, M. C., Gradilone, S. A., Splinter, P., La Russo, N. F., and Marrelli, R. A. (2001) *J. Biol. Chem.* **276**, 12147–12152
33. Brown, D. (2003) *Am. J. Physiol.* **284**, F893–F901
34. Boassa, D., and Yool, A. J. (2002) *Trends Pharmacol. Sci.* **23**, 558–562
35. Richter-Landsberg, C., and Jastorff, B. (1985) *J. Neurochem.* **45**, 1218–1222
36. Sanchez, C., Diaz-Nido, J., and Avila, J. (2000) *Prog. Neurobiol.* **61**, 133–168
37. Diviani, D., and Scott, J. D. (2001) *J. Cell Sci.* **114**, 1431–1437
38. Gull, K. (1999) *Annu. Rev. Microbiol.* **5**, 629–655
39. Schekman, R. (1998) *Nature* **396**, 514–515
40. Merz, A. J., and Wickner, W. T. (2004) *J. Cell Biol.* **164**, 195–206
41. Peters, C., Bayer, M. J., Bühler, S., Andersen, J. S., Mann, M., and Mayer, A. (2001) *Nature* **409**, 581–588
42. Kan, J. P. Y. (1984) *Methods Cell Biol.* **40**, 155–181
43. McManna, J. A. (1973) *Science* **179**, 88–90
44. Linder, J. C., and Stahl, H. L. (1979) *J. Cell Biol.* **83**, 371–382
45. Heuser, J., Zhu, Q., and Clarke, M. (1993) *J. Cell Biol.* **121**, 1311–1327
46. Fok, A. K., Alhara, M. S., Ishida, M., Nolta, K. V., Steck, T. L., and Allen, R. D. (1995) *J. Cell Sci.* **108**, 3163–3170
47. Allen, R. D. (2000) *BioEssays* **22**, 1035–1042
48. Zhu, Q., and Clarke, M. (1992) *J. Cell Biol.* **118**, 347–358
49. Bowers, B., and Korn, E. D. (1973) *J. Cell Biol.* **59**, 784–791
50. Nolta, K. V., and Steck, T. L. (1994) *J. Biol. Chem.* **269**, 2225–2233
51. Nolta, K. V., Padh, H., and Steck, T. L. (1991) *J. Biol. Chem.* **266**, 18318–18323
52. Taylor, M. C., Muhia, D. K., Baker, D. A., Mondragon, A., Schaap, P. B., and Kelly, J. M. (1990) *J. Biol. Chem.* **265**, 205–217
53. Mills, I., Letsou, G., Rabban, J., Sumpio, B., and Gerwitz, H. (1990) *Biochem. Biophys. Res. Commun.* **171**, 143–147
54. Martinac, B. (2004) *J. Cell Sci.* **117**, 2449–2460
55. Martines, B., Wang, Y., Benaim, G., Benchimol, M., de Souza, W., Scott, D. A., and Docampo, R. (2002) *Mol. Biochem. Parasitol.* **120**, 205–213
56. Merzendorfer, H. M., Graf, R., Huss, M., Harvey, W. R., and Wieczorek, H. (1997) *J. Exp. Biol.* **200**, 225–235
57. Moriyama, Y., and Nelsen, N. (1989) *J. Biol. Chem.* **264**, 3577–3582
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Peter Rohloff, Andrea Montalvetti and Roberto Docampo

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