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Stress response to high osmolarity in *Trypanosoma cruzi* epimastigotes

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ABSTRACT

Trypanosoma cruzi undergoes differentiation in the rectum of triatomine, where increased osmolarity is caused mainly by elevated content of NaCl from urine. Early biochemical events in response to high osmolarity in this parasite have not been totally elucidated. In order to clarify the relationship between these events and developmental stages of *T. cruzi*, epimastigotes were subjected to hyperosmotic stress, which caused activation of Na^+/H^+ exchanger from acidic vacuoles and accumulation of inositol trisphosphate ($InsP_3$). Suppression of $InsP_3$ levels was observed in presence of intracellular Ca^{2+} chelator or pretreatment with 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), which also inhibited the alkalinization of acidic vacuoles via a Na^+/H^+ exchanger and the consequent increase in cytosolic calcium. These effects were activated and inhibited by PMA and Chelerythrine respectively, suggesting regulation by protein kinase C. The *T. cruzi* Na^+/H^+ exchanger, TcNHE1, has 11 transmembrane domains and is localized in acidic vacuoles of epimastigotes. The analyzed biochemical changes were correlated with morphological changes, including an increase in the size of acidocalcisomes and subsequent differentiation to an intermediate form. Both processes were delayed when TcNHE1 was inhibited by EIPA, suggesting that these early biochemical events allow the parasite to adapt to conditions faced in the rectum of the insect vector.

Introduction

The flagellate protozoan *Trypanosoma cruzi* is responsible for Chagas' disease, a serious endemic illness prevalent throughout Latin America. This parasite has a complex life cycle involving several morphological and functionally different stages that adapt to a variety of conditions imposed by the insect vector and mammalian host environments. The ability of *T. cruzi* to receive signals from different environments and to initiate appropriate changes in cell activity is crucial for its pathogenic activity. Previous evidences demonstrated that the parasite is able to respond to different agonists by activation of a phosphatidylinositol 4,5 bisphosphate specific phospholipase C (PI-PLC)[3] [1,2], and consequently the inositol phosphate pathway [3,4]. *T. cruzi* PI-PLC showed an absolute dependence on Ca^{2+} for its activity [5,6]. This enzyme contains amino acid residues important for binding of $InsP_3$ and Ca^{2+} in the active site, as well as putative Ca^{2+} ligands in the C2 domain. It is known that calcium signaling is required at several points in the life cycle of *T. cruzi*, including host cell invasion [7], multiplication, and differentiation [8].

Most of the releasable Ca^{2+} in trypanosomatids and apicomplexan parasites is contained in acidocalcisomes which are acidic vacuoles that possess a Ca^{2+}/nH^+ exchanger, and several pumps [9]. In situations in which cells are exposed to NaCl stress, the yeasts and plants have developed several mechanisms to maintain low levels of salt in the cytoplasm, including removal of sodium by transport out of cell and/or into the vacuoles by Na^+/H^+ exchanger activation [10]. *T. cruzi* acidocalcisomes share several properties with vacuoles of plants, and a potential osmoregulatory function of these acidic organelles [11]. In previous work [12], we showed dependence between extracellular Na^+ and Ca^{2+} release from intracellular store evoked by Carbachol. In this context, the slow component of Ca^{2+} signaling induced by the agonist was reduced in a Na^+-free medium and progressively increased when the extracellular pH raised, indicating that Ca^{2+} signaling was modified by a driving force imposed by opposite Na^+ and H^+ gradients. Thus, we suggested a model in which Na^+ and H^+ extracellular may play an important regulatory role in allowing the phosphoinositide cycle to proceed in the parasite response to certain extracellular signals. Moreover, Gimenez et al. [13] demonstrated that high osmolarity during epimastigote growth leads to intermediate forms, which showed PI-PLC activity higher

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[3] *Abbreviations used:* AO, Acridine Orange; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; $[Ca^{2+}]_i$, intracellular free calcium concentration; $InsP_3$, inositol trisphosphate; KRT, Krebs–Ringer–Tris; NMG, N-methyl-D-glucamine; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate ester; PKC, protein kinase C; BCECF, 2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein.

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than that of epimastigotes. These parasite forms, considered to be a pre-adaptation of epimastigotes for differentiation to tripomastigotes [14], were also found in the alimentary tract of reduviid insect where epimastigotes propagate [15,16]. The environmental pH and Na^+ concentration vary considerably in the triatomine gut and as a result, the osmolarity increases sharply to 600–1000 mOsm/l. However, an important question remaining is how the increased osmolarity is transmitted into the parasite. Therefore, we studied the relationship between increased osmolarity of the medium and phosphatidylinositol pathway in epimastigotes of *T. cruzi*, mimicking the situation that the parasites encounter in the insect's rectum where metacyclogenesis takes place. In this context, we found a Na^+/H^+ exchanger involvement in the PLC activation process, via stimulation by protein kinase C and cytosolic calcium increase. Furthermore, we also show analysis of the amino acid sequence, phylogenetic position and strong evidence that Na^+/H^+ exchanger is localized in acidic vacuoles of *T. cruzi* epimastigotes.

Materials and methods

Cells and culture media

The *T. cruzi* Tulahuen strain was used in this study. Epimastigote forms were grown at 28 °C in culture medium supplemented with 10% fetal bovine serum (FBS), as described previously by Machado de Domenech [1]. Cells in the logarithmic phase of growth were harvested by centrifugation at 1500*g* for 10 min and washed twice with 25 mM Tris–HCl, pH 7.35, 1.2 mM $MgSO_4$, 2.6 mM $CaCl_2$, 4.8 mM KCl, 120 mM NaCl, and 100 mM glucose [Krebs–Ringer–Tris (KRT) buffer].

Measurement of alkalinization in acidic vesicles and epifluorescence microscopy

The alkalinization of vesicles in intact epimastigotes was assayed by measuring changes in absorbance of Acridine Orange (AO) [17] using a GenesisTM spectrophotometer (Spectronic®, Milton Roy Company) at the wavelength pair 493–530 nm, described by Bollo et al. [12] and a spectrofluorometer Fluoromax 3 at excitation and emission wavelength of 493 and 530 nm, respectively.

Briefly, cells harvested in the logarithmic phase were suspended in 1.5 ml of KRT buffer (5×10^7 cells/ml), incubated with 10 μM AO for 30 s o 15 min, and added with various effectors at 28 °C. In all cases, cells were preincubated with 1 μM 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) for 10 min prior to the addition of effectors. For experiments under Na^+-free conditions, NaCl was replaced by 120 mM N-methyl-D-glucamine (NMG).

For epifluorescence microscopy experiments, parasites were resuspended in KRT buffer plus AO (10 μM) for 15 min at 28 °C, and washed twice in KRT buffer to eliminate excess of dye. Parasites were then treated with various effectors for 15 min at 28 °C, and 10 μl aliquots were placed on the coverslips and observed under epifluorescence microscope (Zeiss AxioLab Standard Fluorescence Microscopy) fitted with a filter set 09 (Zeiss), excitation 450–490 nm and emission 510 nm.

Measurement of $[Ca^{2+}]_i$ with Fura 2-AM

Epimastigotes were harvested and washed once in KRT buffer. $[Ca^{2+}]_i$ was determined as described previous [12]. Briefly, cells were resuspended in KRT buffer (2×10^8 cells/ml), incubated with 4 μM Fura 2-AM in the dark for 60 min at 28 °C in a water bath with mild agitation, washed twice with ice-cold KRT buffer, incubated for 20 min at 28 °C with agitation, and kept in ice in the dark until use. For fluorescence measurement, a 0.375 ml aliquot of Fura 2-loaded epimastigote suspension was diluted into 1.5 ml KRT buffer (final concentration 5×10^7 cells/ml), and placed in polystyrene cuvets. Fluorescence was recorded in a DM3000 spectrofluorimeter (SPEX Industries, Edison, NJ, USA) equipped with a thermostated (28 °C) cuvet holder and a stirring device. Excitation and emission wavelengths were 340 and 500 nm, respectively.

Normalized fluorescence values were determined as described [18]. Calcium release in response to effectors was determined by integrating the total fluorescence signal obtained [19]. This value shows the amount of calcium released, relative to the area under the transient curve for control, defined as 100%.

Measurements of acidification in the cytoplasm

Cytoplasmic acidification in intact epimastigotes was determined by changes in the fluorescence of 2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein (BCECF), a pH indicator. The experiments were carried out in spectrofluorometer Fluoromax 3 with excitation wavelengths of 440 and 500 nm, and with emission wavelength of 530 nm.

Parasites were suspended in KRT buffer-glucose-sulfinpyrazone (glucose 1.8% w/v -sulfinpyrazone 200 μM) pH 7.2. The cells were stabilized for 20 min, then added 9 μM BCECF and incubated in the dark at 28 °C for 60 min. For measurements, 5×10^7 cells/ml in a final volume of 1.5 ml were loaded into cuvettes thermostatically controlled at 28 °C and the different effectors were added. The cells were pre-incubated with 1 μM EIPA for 10 min prior to addition of effectors.

Confocal microscopy

Epimastigotes were harvested and washed twice with KRT buffer. A 10 μl aliquot (4×10^6 cells/ml) were fixed with methanol at −20 °C for 6 min, washed with PBS and incubated with PBS-albumin for 30 min at room temperature. Parasites were then incubated with anti-Na^+/H^+ and anti-VH^+ PPase, dissolved in PBS 1% v/v for 90 min, washed three times with PBS for 5 min and then incubated for 60 min with secondary antibodies anti-goat IgG FITC labeled and anti-rabbit IgG Rhodamine labeled, both dissolved in PBS 1% v/v. The cells were observed by confocal microscopy Nikon Eclipse C1si spectral excitation with Argon laser line 488 for FITC and He-Ne laser 543 for Rhodamine, respectively.

Electron microscopy

Parasites were pre-incubated and gently agitated in a shaking water-bath for 10 min at 28 °C, in KRT buffer plus 0.1% bovine serum albumin (BSA), treated with 0.5 M NaCl or 1 M mannitol, added with ice-cold KRT buffer, and centrifuged at 1000*g* for 5 min. The pellet was fixed with 2% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer pH 7.2 (buffer A) for 1 h. The pellet was washed twice with buffer A, fixed with 1% osmium tetroxide in buffer A, washed twice with water, dehydrated with increasing acetone concentrations (up to 100%), and embedded in epoxy resin (Araldite) for 24 h at 60 °C. Thin sections were cut by automated precision ultramicrotome and observed in a LEO 906 transmission electron microscope (Zeiss, Oberkochen, Germany). Vesicle diameter was measured in a significant number of cells (at least 100 for each condition).

Determination of morphological changes

Epimastigotes were suspended in sterile KRT buffer (5×10^7 cells/ml) and stabilized at 28 °C. Then, the different effec-