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Ca^{2+} transport in isolated mitochondrial vesicles from *Leishmania braziliensis* promastigotes

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Leishmania braziliensis maintained very low (50±20 nM) intracellular concentrations of calcium ions under normal conditions, as shown by the fluorimetric indicator QUIN2. Digitonin-permeabilized cells liberated large amounts of calcium ions in the presence of the ionophore A23187, indicating the presence of a large intracellular reservoir for this ion. Given the extraordinary extension of the single giant mitochondrion of Kinetoplastida and the known capacity of mitochondria from other sources to accumulate calcium, we tested the capacity of this organelle to accumulate calcium ions in *Leishmania*. Coupled mitochondrial vesicles, five-fold enriched in succinate-cytochrome *c* oxidoreductase, were obtained from promastigotes by gentle grinding (45 s) with glass beads in hypertonic buffer solution, followed by differential centrifugation. These vesicles had a respiratory control ratio of 1.82±0.15, and two phosphorylation sites (sites II and III) using succinate as electron donor, and were capable of calcium uptake in the presence of several respiratory substrates; this uptake was enhanced in the presence of ADP and P_i and was blocked by classical electron transport inhibitors. Uncouplers such as carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) and the calcium ionophore A23187 released previously accumulated calcium ions, suggesting that the driving force for the calcium uptake by the vesicles is the respiratory generated electrochemical potential gradient of protons. A study of the affinity of this system for calcium showed that even at 90μM free calcium, succinate-induced calcium uptake is not saturated while approaching a level of 200 nmol min^{-1} (mg protein)$^{-1}$, indicating a low-affinity, large-capacity system. These results indicate the the *Leishmania* mitochondrion is involved in calcium ion homeostasis in these cells; not in the maintenance of the basal cytoplasmic levels, but most probably in the recovery from large fluctuations.

Key words: *Leishmania braziliensis*; Kinetoplastida; Ca^{2+} homeostasis; Mitochondrion; Metallochromic calcium indicator; Fluorimetric calcium indicator

Introduction

Calcium ions have been recognized as mediators in a large number of cellular processes [1]. In protozoan parasites of the Trypanosomatidae family, calcium has been implicated in microtubule assembly [2], flagellar movement [3], release of variable surface antigens [4], differentiation processes [5] and as modulators of adenyl cyclase and cAMP-dependent phosphodiesterase [7]. Calmodulin has also been found in these parasites [7–10], and phenothiazine drugs, which are well-known calmodulin antagonists, are highly cytotoxic to *Leishmania* [11] and African trypanosomes [12]. Finally, alteration of calcium homeostasis seems to be the basis for the lytic effect of drugs that block energy metabolism in the bloodstream forms of African trypanosomes [13]. All these facts point directly to the relevance of the study of the mechanisms of calcium homeostasis in these organisms, as it seems to be essential for its parasitic function. However, very little

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenendiamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethylene glycol bis-(b-aminoethylether)-*N,N′*-tetraacetic acid; QUIN2, 2-[(2-bis-[carboxymethyl]amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis[carboxymethyl]aminoquinoline; QUIN2-AM, 2-[(2-bis-[carboxymethyl] amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis[carboxymethyl] aminoquinolinetetrakis-[acetoxymethyl]ester.

is known concerning these mechanisms in Kinetoplastida or in protozoans in general.

In higher eukaryotic cells calcium homeostasis is achieved by several processes: calcium extrusion by the Ca^{2+}-Mg^{2+} ATPase, Ca^{2+}/Na^{+} exchange at the plasma membrane, and calcium uptake and release from the mitochondrial and endoplasmic reticulum compartments [14]. Given the characteristic single giant mitochondrion of the kinetoplastid protozoans, we set out to investigate the possible involvement of this organelle in the calcium regulation of *Leishmania braziliensis* promastigotes. In other cells the most commonly accepted hypothesis for the mechanism of intramitochondrial calcium accumulation points to the difference in the proton electrochemical potential across the internal mitochondrial membrane as the driving force which energizes an active calcium uptake through a unidirectional electrophoretic uniporter; the release mechanism is thought to be an independent Na^{+}/Ca^{2+} exchange present in the same membrane (see ref. 15 for a general review).

In this report we present evidence for the involvement of the mitochondrial compartment in the regulation of the cytoplasmic concentration of calcium in *Leishmania* promastigotes, inferred from the capacity of isolated mitochondrial vesicles to transport and accumulate this cation coupled to the generation of a proton electrochemical potential gradient.

Materials and Methods

Organism. The Lby stock of *L. braziliensis* [16] (concerning the current taxonomic status of the isolate, see ref. 17) was grown in liver infusion-tryptose (LIT) medium at 26°C, with strong agitation (120 rev./min).

Measurements of calcium movements in whole permeabilized promastigotes. Exponential phase promastigotes were harvested by centrifugation at 1000 × *g* for 10 min washed twice in buffer A (140 mM NaCl/11 mM KCl/75 mM Tris-HCl, pH 7.4) and resuspended in the same buffer at a final density of 5×10^8 cells ml^{-1}, in the presence of 100 μM Arsenazo III, a non-permeant metallochromic indicator which is highly selective for free calcium ions under the experimental conditions chosen in this work [18]. Digitonin was added to a final concentration of 100 μg ml^{-1} and calcium movements were followed by measuring the changes in the absorbance spectrum of the metallochromic indicator using an Aminco DW-2a dual wavelength spectrophotometer. The wavelength pair chosen (664–696 nm) gave optimal sensitivity and selectivity under these conditions.

Determination of intracellular calcium concentrations. Washed promastigotes were incubated in buffer A plus 10 mM glucose (3×10^8 cells ml^{-1}) containing 50 μM of the acetoxymethyl derivative of QUIN2 (QUIN2-AM) for 1–2 h at 26°C. The cells were then washed twice by centrifugation and resuspended in the same glucose-containing buffer without the fluorochromic indicator, at the above indicated cell density. QUIN2-AM is readily permeable and is transformed to the impermeant compound QUIN2 by the sterases present in the cell's cytoplasm; QUIN2 fluoresces upon binding of (free) calcium ions [19]. The total fluorescence of the cell suspension (excitation 339 nm, emission 492 nm) was measured using an Aminco-Bowman spectrophotofluorometer and the intracellular ionic calcium concentration calculated according to the following equation [19]:

$$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F)$$

Where K_d is the dissociation constant for the QUIN2-Ca^{2+} complex (115 nM), F is the measured fluorescence intensity under some given conditions, F_{min} the fluorescence for parasites in the presence of 4 mM EGTA ($[Ca^{2+}] < 1$ nM) and F_{max} the fluorescence in the presence of digitonin and excess Ca^{2+} (1 mM).

Subcellular fractionation. An enriched mitochondrial fraction was obtained by a modification of a method described previously for the preparation of plasma membranes from *Leishmania mexicana* [20] and *Trypanosoma cruzi* [21]. Briefly, promastigotes (10^{11} cells) were harvested and washed as described above; after a final wash in buffer B (400 mM mannitol/10 mM KCl/1 mM magne-

sium acetate/10 mM Hepes/soybean trypsin inhibitor 0.15 mg ml^{-1}/1 mM PMSF, pH adjusted to 7.4 with KOH) the cell pellet was mixed with acid-washed glass beads (75–120 μm diameter, Sigma) at a ratio of 1:4 (wet weight/weight of beads) and the cells were disrupted by abrasion in a chilled mortar for 45–60 s. Beads, unbroken cells and large cell debris were removed by centrifugation at 1000 × *g* for 15 min, and the enriched mitochondrial fraction was separated by centrifugation at 5000 × *g* for 20 min at 4°C. The pellet was gently resuspended in buffer B and immediately used for respiration and calcium transport experiments.

Respiration experiments. Oxygen uptake by the enriched mitochondrial fraction (0.8–1.2 mg ml^{-1}) was followed polarographically with a Yellow Spring Instruments respirometer at 26°C in buffer B, supplemented with 5 mM KH_2PO_4 and 2 mM magnesium acetate.

Measurement of calcium transport by the mitochondrial subcellular fraction. Net calcium fluxes in the presence of the enriched mitochondrial fraction (0.6–0.8 mg ml^{-1}) were measured in buffer C (200 mM mannitol/110 mM KCl/1 mM magnesium acetate/10 mM sodium succinate/5 mM KH_2PO_4/0.5 mM ADP/20 mM Hepes/1 mM EGTA pH 7.4 adjusted with KOH) at 26°C by two different methods: (a) spectrophotometrically, using Arsenazo III (50 μM) as described above for whole cells, but the wavelength pair was 675–685 nm to avoid interference by Mg^{2+} present in the assay medium, and (b) by measuring the radioactivity retained in the subcellular fraction after incubation in the presence of 2 nmol of $^{45}Ca^{2+}$ per ml with an specific activity of 100 cpm $pmol^{-1}$ in buffer C; to this medium variable amounts of $CaCl_2$ were added to obtain the desired ionic calcium concentration, calculated as described by Fabiato and Fabiato [22]. The assay was started by addition of the mitochondrial fraction and stopped after various times by rapid filtration through nitrocellulose filters (0.45 μm mean pore diameter, Millipore). Non-specific absorption was corrected for by subtracting the radioactivity retained in the filters at time zero.

Enzyme assays. Succinate-cytochrome *c* oxidoreductase was measured using horse heart cytochrome *c*, according to Sotocasa et al. [23] at 26°C. Protein was determined according to Lowry et al.[24], using bovine serum albumin as standard.

Materials. A23187 was a gift from Eli Lilly and Company (Venezuela). Arsenazo III, digitonin, QUIN2-AM and all other biochemicals were products of Sigma-Aldrich Corporation. Other reagents were of the highest purity available and were purchased from Merck. $^{45}Ca^{2+}$ (100 dpm $pmol^{-1}$) was provided by New England Nuclear Corporation.

Results

The intracellular ionic calcium concentration of *L. braziliensis* promastigotes, determined by the fluorimetric calcium indicator QUIN2, is 50±20 nM, which shows the presence of powerful regulatory mechanisms for this ion in these protozoan cells. When the cells were permeabilized by the use of digitonin, a very small calcium release to the medium was observed using Arsenazo III as metallochromic indicator (Fig. 1); however, if addition of the detergent was followed by addition of the calcium ionophore A23187, a large and rapid increase in the medium calcium concentra-

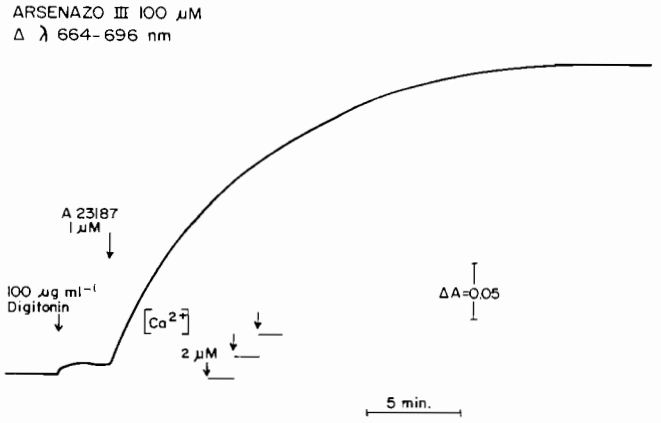

Fig. 1. Calcium release from digitonin-permeabilized *L. braziliensis* promastigotes. Free calcium concentrations were followed in a suspension of promastigotes (10^8 cells ml^{-1}) using the metallochromic indicator arsenazo III (100 μM) and the wavelength pair 664 nm/696 nm, as described in Materials and Methods.

TABLE I

Respiratory and succinate-cytochrome *c* oxidoreductase activities in whole cell homogenates and enriched mitochondrial fraction from *Leishmania braziliensis* promastigotes

Fraction	Respiratory activity (nmol O_2 min^{-1} (mg protein)$^{-1}$)	Succinate-cytochrome *c* oxido-reductase activity (nmol min^{-1} (mg protein)$^{-1}$)
Homogenate	2.7±0.3 (N=5)	0.6±0.2 (N=5)
Mitochondrial fraction	12.9±0.9 (N=5)	2.8±0.8 (N=5)

Respiratory activity was measured polarographically using succinate (10 mM) as electron donor and succinate-cytochrome *c* oxidoreductase spectrophotometrically as described in Materials and Methods, at 26°C. Indicated are mean values and standard errors, N being the number of independent experiments.

tion was found, which was not present if the organic solvent vehicle used for the ionophore was added. This observation suggests the presence of an intracellular compartment with a large capacity for calcium storage. A likely candidate for this compartment in these cells is the single giant mitochondrion, which occupies around 12% of the cell volume [24], as the ability of these organelles to accumulate calcium is well known [15]. To test this hypothesis, we prepared an enriched fraction of mitochondrial particles by gentle abrasion of the cells with glass beads, as described in Materials and Methods. This fraction is five-fold enriched in succinate-cytochrome *c* oxidoreductase compared to whole-cell homogenates, and exhibits a specific respiratory rate proportional to the enrichment of the marker enzyme (Table I). Fig. 2 shows that these particles also display respiratory control, as shown by ADP-induced State 4–State 3 transitions and CCCP-induced irreversible respiratory release (State 3u). The respiratory control ratio (ratio of State 3 to State 4 respiratory rates) using succinate as electron donor is 1.82±0.15 (N=9), while the ADP/O ratio is 1.92±0.31 (N=6), indicating the presence of two phosphorylating sites (sites II and III). A demonstration that the respiration enhancement produced by ADP is coupled to phosphorylation is given by the blockade of the State 4–State 3 transition by oligomycin, a specific inhibitor of the mitochondrial F1-F0 ATP synthetase (Fig. 2). Further proof for the presence of predominantly right-side-out sealed mitochondrial vesicles in this preparation were the effects of oxaloacetate and malonate, both strong competitive inhibitors of particulate and soluble succinate dehydrogenase, on succinate (10 mM)-induced respiration; while oxaloacetate at 0.5 mM has only marginal effects (<10%), malonate at 5 mM blocks respiration by 90% (results not shown). This result clearly shows that the active site of the enzyme is not accessible to the impermeant oxaloacetic acid, but can be reached by malonate, which permeates the inner mitochondrial membrane [26]. Finally, electron transport from proline, succinate and ascorbate (in the presence of TMPD) to oxygen is inhibited by classical respiratory blockers (Table II).

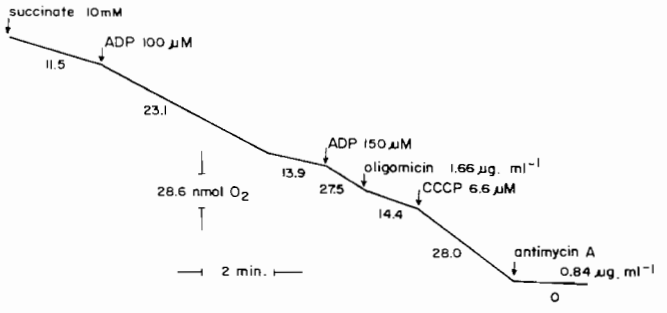

Fig. 2. Respiratory activity of an enriched mitochondrial fraction obtained from *L. braziliensis* promastigotes. Oxygen consumption was followed polarographically in a suspension (1.1 mg ml^{-1} in buffer C) of an enriched preparation of mitochondrial vesicles as described in Materials and Methods. Respiratory rates are expressed as nmol O_2 min^{-1} (mg protein)$^{-1}$.

The capacity of the mitochondrial fraction for calcium transport was determined using Arsenazo III as an indicator for ionic calcium. As can be observed in Fig. 3, upon addition of succinate there was calcium uptake by the mitochondrial vesicles which tended to level off after approximately 5 min. The driving force for this uptake was the generation of a proton electrochemical gradient by the vesicles as can be seen by the release of the accumulated calcium by addition of

TABLE II

Effects of electron-transport blockers on the oxygen consumption of an enriched mitochondrial fraction from *Leishmania braziliensis* promastigotes[a]

Inhibitor	Respiratory substrate	% Inhibition of respiration
Rotenone, 20 μM	L-Proline, 10 mM	48.6±7.1 (N=3)
Rotenone, 30 μM	L-Proline, 10 mM	60.2±2.4 (N=3)
Rotenone, 50 μM	L-Proline, 10 mM	90.3±1.7 (N=3)
Antimycin A, 0.42 μg ml^{-1}	Succinate, 10 mM	88.2±3.6 (N=8)
Antimycin A, 0.84 μg ml^{-1}	Succinate, 10 mM	100 (N=7)
Antimycin A, 0.42 μg ml^{-1}	L-Proline, 10 mM	100 (N=3)
KCN, 0.5 mM	Succinate, 10 mM	82.0±0.5 (N=4)
KCN, 1 mM	Succinate, 10 mM	100 (N=3)
KCN, 1 mM	Ascorbate, 1 mM[a]	100 (N=3)
NaN_3, 2 mM	Succinate, 10 mM	38.1±6.2 (N=3)
NaN_3, 8 mM	Succinate, 10 mM	70.6±7.1 (N=3)

Oxygen consumption by the enriched mitochondrial fraction (0.8–1.2 mg ml^{-1}) was measured polarographically at 26°C, as described in Materials and Methods. Indicated are means and standard errors for the percentual inhibition produced by the electron transport blocker, in steady state. N indicates the number of independent experiments.

[a]In the presence of 50 μM TMPD and 0.42 μg ml^{-1} of antimycin A.

the uncoupler FCCP; the FCCP-induced release of calcium, however, was slower than that observed with the calcium ionophore A-23187, although both reached the same level if sufficient time was allowed (data not shown). It is worth noting that the level of calcium in the medium reached after addition of the ionophore was higher than the starting level (Fig. 3), showing that the mitochondrial vesicles contained stored calcium.

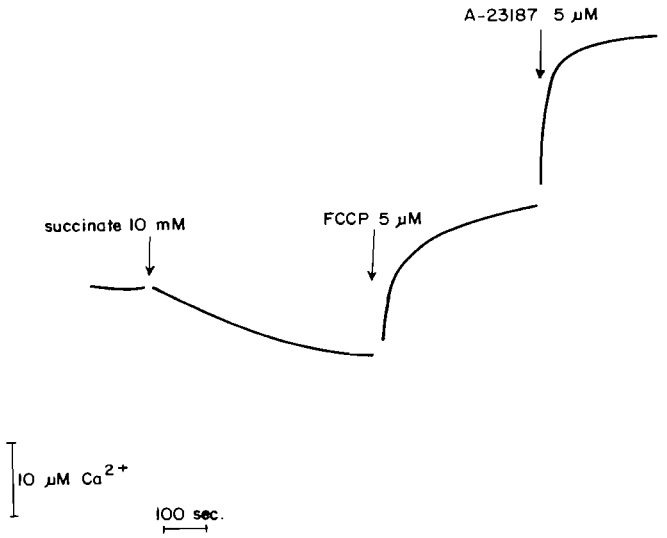

Fig. 3. Calcium uptake and release from an enriched mitochondrial fraction obtained from *L. braziliensis* promastigotes. Free calcium concentrations were followed using the metallochromic indicator arsenazo III (50 μM) and the wavelength pair 675 nm/685 nm, as described in Materials and Methods. Protein concentration of the mitochondrial fraction was 0.6 mg ml^{-1}; the vesicles were incubated in buffer C without ADP and KH_2PO_4 and the indicated additions.

As shown for other mitochondrial calcium transport systems [15] the succinate-induced calcium uptake in *Leishmania* submitochondrial vesicles is strongly enhanced by the presence of phosphate and ADP in the assay medium (Fig. 4); furthermore, these two substances alone were able to induce calcium uptake, demonstrating the presence of respiratory substrates inside the vesicles. The link between calcium transport and cytochrome-mediated respiratory activity was shown by its total inhibition by 1 μg ml^{-1} antimycin A; the calcium uptake in antimycin A poisoned vesicles was partially activity by the couple ascorbate/TMPD and was finally abolished by 0.5 mM cyanide.

To characterize the calcium affinity of the *Leishmania* mitochondrial transport system, $^{45}Ca^{2+}$ uptake was measured as a function of total ionic calcium concentration in the assay medium, buffered with EGTA (Fig. 5). Although the

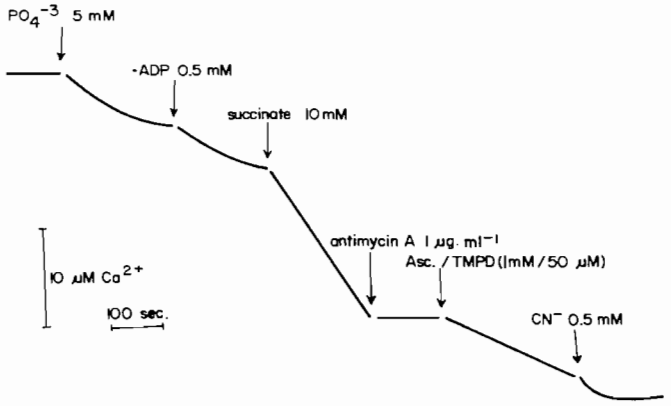

Fig. 4. Effect of respiratory substrates and electron transport blockers on calcium uptake by an enriched mitochondrial fraction obtained from *L. braziliensis* promastigotes. Experimental details as described for Fig. 3. Protein concentration of the mitochondrial fraction was 0.8 mg ml^{-1}.

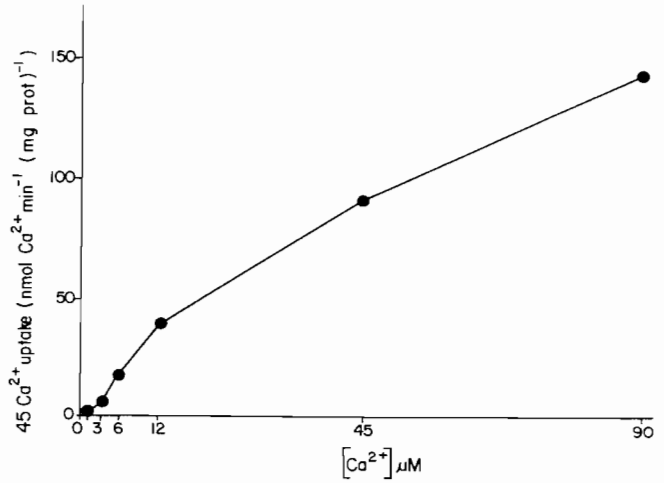

Fig. 5. Dependence of calcium uptake by an enriched mitochondrial fraction obtained from *L. braziliensis* promastigotes on free ionic calcium concentration. $^{45}Ca^{2+}$ uptake by the mitochondrial fraction (0.8 mg ml^{-1} in buffer C) was measured by a rapid filtration method as described in Materials and Methods.

process is obviously saturable, complete saturation was not observed even with 90 μM free ionic calcium, under the experimental conditions chosen; at this level the uptake rate approaches 200 nmol min^{-1} (mg protein)$^{-1}$.

Discussion

The ionic cytoplasmic calcium concentration measured in this work for *Leishmania* promastigotes is comparable with that reported for other eukaryotic cells [27], which indicates the presence of homeostatic mechanisms of similar efficiency. One well known mechanism for this regulation is the calcium buffering ability of mitochondria. The results obtained both in digitonin-permeabilized cells and isolated mitochondrial vesicles strongly suggest that the single mitochondrion found in these cells is involved in the storage and regulation of calcium ions. The respiratory properties of these vesicles (Fig. 2), including respiratory control and oxidative phosphorylation, are comparable to those reported for other coupled mitochondrial preparations from Trypanosomatids [26,28–30] and agree with those found in previous studies on both intact cells and mitochondrial fractions from *Leishmania* [31–33]. The dependence of the calcium uptake on respiratory substrates and the effects of classical electron transport blockers support the proposition that calcium ions are transported solely by mitochondrial vesicles in this preparation. In addition, as shown for other mitochondrial systems, the driving force for calcium uptake is the respiration-dependent gradient of proton electrochemical potential. This function could be of great survival value when these cells proliferate inside the parasitophorous vacuoles or cytoplasm of the host's cells, where the free calcium concentration is extremely low. As indicated in the Introduction, calcium ions seem to perform essential roles in the parasite cell, probably related to the assembly of sub-pellicular microtubules [2] and flagellar function [3], as well as its possible role as second messenger [6–10]. The notion of important intracellular functions of calcium ions in *Leishmania* is supported by the presence of large amounts of calmodulin reported by us in these cells [10] and by others in related members of the Trypanosomatidae family [7–9]. However, it is clear from the low basal levels of cytoplasmic ionic calcium and the relatively low affinity of the mitochondrial calcium transport system described here that this system cannot be responsible for the fine tuning of ionic calcium levels around its resting value; it might be involved in the recovery of basal levels from large fluctations which could arise from flagellar movements. The relative po-

sition of flagellum and mitochondrion in these cells is consistent with a tight functional coupling of these organelles. Also consistent with this interpretation is the recent finding of a plasma membrane-associated Ca^{2+}-Mg^{2+}-dependent ATPase which vectorially transports calcium with high affinity in *Leishmania* promastigotes (Benaim, G., in preparation).

Recently, Docampo and Vercesi [34,35] have presented results for digitonin-permeabilized *Trypanosoma cruzi*-epimastigotes that indicate that the mitochondrial compartment is also involved in the cytoplasmatic calcium regulation in this other trypanosomatid; however, the present work is the first direct demonstration of calcium transport ability of an isolated mitochondrial preparation from these parasites. Starting from this preparation it should be possible to characterize the molecular mechanisms involved in this transport process without the interference of other cellular systems involved in the regulation of this cation.

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