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Ca^{2+} transport by digitonin-permeabilized *Leishmania donovani*

Effects of Ca^{2+}, pentamidine and WR-6026 on mitochondrial membrane potential *in situ*

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The use of low concentrations of digitonin allowed the quantitative determination of the mitochondrial membrane potential of *Leishmania donovani* promastigotes *in situ* using safranine O. *L. donovani* mitochondria were able to build up and retain a membrane potential of a value comparable with that of mammalian mitochondria. The response of promastigotes mitochondrial membrane potential to phosphate, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), valinomycin and Ca^{2+} indicates that these mitochondria behave similarly to vertebrate mitochondria with regard to the properties of their electrochemical proton gradient. When *L. donovani* promastigotes were permeabilized with digitonin in a reaction medium containing MgATP, succinate and 3.5 μM free Ca^{2+}, they lowered the medium Ca^{2+} concentration to the submicromolar level (0.05–0.1 μM). The presence of 1 μM-FCCP decreased by about 75% the initial rate of Ca^{2+} sequestration by these permeabilized cells. This FCCP-insensitive Ca^{2+} uptake, probably by the endoplasmic reticulum, was completely inhibited by 500 μM-vanadate. On the other hand, when vanadate instead of FCCP was present, the initial rate of Ca^{2+} accumulation was decreased by about 25% and the Ca^{2+} set point was increased to 0.7 μM. The succinate-dependence and FCCP- and Ruthenium Red-sensitivity of the Ca^{2+} uptake detected in the presence of vanadate indicate that this uptake is probably by the mitochondria. This interpretation was further supported by the Ruthenium Red-sensitive decrease in the mitochondrial membrane potential caused by Ca^{2+} addition. The anti-leishmanial cationic drugs pentamidine and WR-6026 also induced a rapid collapse of the mitochondrial inner membrane potential of *L. donovani* promastigotes.

INTRODUCTION

We have previously shown [1,2] that digitonin-permeabilized *Trypanosoma cruzi* epimastigotes, when incubated in a reaction medium containing MgATP, lowered the Ca^{2+} concentration to a submicromolar level at the expense of two intracellular Ca^{2+}-transporting activities: the electrophoretical Ca^{2+} uniporter present in the mitochondrial inner membrane, and an ATP-dependent, vanadate-sensitive mechanism, probably represented by the endoplasmic reticulum. The former showed a faster rate of Ca^{2+} uptake at high Ca^{2+} levels ($[Ca^{2+}] > 1$ μM) but a much lower affinity (Ca^{2+} set point close to 0.7 μM) than the non-mitochondrial activity [2]. As has been demonstrated with other types of eukaryotic cells studied [3], we have observed that despite the lower capacity for Ca^{2+} accumulation, its relatively high affinity for Ca^{2+} as compared with mitochondria indicates that the endoplasmic reticulum is responsible for the buffering activity at the low cytosolic Ca^{2+} levels present *in situ* [2]. Additional studies on the operation of intracellular Ca^{2+} pools in *T. cruzi* and *T. brucei* (A. Vercesi & R. Docampo, unpublished work) have indicated that mitochondria *in situ* contain none or only a very small fraction of the endogenous cellular Ca^{2+}. This, in conjunction with their low affinity for the cation, indicates that these organelles, similarly to vertebrate mitochondria [3,4], are not involved in the regulation of the cytosolic Ca^{2+} concentration under normal physiological conditions.

With regard to the regulation of calcium homeostasis in other protozoa, an interesting work [5] has suggested that in *Leishmania donovani* promastigotes two intracellular pools, the mitochondrion and the endoplasmic reticulum, are involved in the regulation of the cytosolic free Ca^{2+} concentration. Although these authors performed a meticulous characterization of the non-mitochondrial Ca^{2+} transport activity in these cells, the lack of data on the properties of Ca^{2+} transport by mitochondria left the question of the relationship between those intracellular Ca^{2+} pools largely unresolved. The objective of the present study was to re-examine the respective roles and relationships between mitochondrial and non-mitochondrial Ca^{2+} transport activities in *L. donovani* promastigotes, as well as to further characterize the mechanism of mitochondrial Ca^{2+} transport. Therefore the safranine technique [6,7] was used to estimate the changes in mitochondrial membrane potential associated with Ca^{2+} accumulation by the organelle. The use of this technique also permitted the verification that the cationic anti-leishmanial drugs pentamidine and WR-6026 have a potent uncoupling effect on these mitochondria *in situ*.

MATERIALS AND METHODS

Culture methods

L. donovani promastigotes (S-2 strain) were grown at 28 °C in a liquid medium consisting of Minimum Essential Medium Eagle (MEM) (Sigma M3024) supplemented with 30 mM-Na-Hepes buffer (pH 7.3), 2 mM-glutamine, 26 mM-$NaHCO_3$, 5 mM-proline, 2 mM-sodium citrate, 27 mM-glucose, haemin (7.5 mg/l) and 10% heat-inactivated fetal calf serum. At 3–4 days after inoculation, the cells were collected by centrifugation (1500 *g*, 5 min) and washed twice in a buffer containing 116 mM-NaCl, 5.4 mM-KCl, 0.8 mM-$MgSO_4$, 5.5 mM-D-glucose and 50 mM-Hepes at pH 7.0. The final concentration of cells was determined using a Neubauer chamber. The protein concentration was determined by the biuret assay in the presence of 0.2% deoxycholate [8].

Abbreviations used: $\Delta\Psi$, mitochondrial membrane potential; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; WR-6026, 8-(6-diethylaminohexylamino)-6-methoxy-4-methylquinoline dihydrochloride.

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Chemicals

ATP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), pentamidine, valinomycin, Ca^{2+} ionophore A23187, sodium orthovanadate, safranine O, Arsenazo III, EGTA, haemin, Hepes, EGTA, fetal calf serum, Ruthenium Red and digitonin were purchased from Sigma. 8-(6-Diethylaminohexylamino)-6-methoxy-4-methylquinoline dihydrochloride (WR-6026) was a gift from the Walter Reed Army Institute (Washington, DC, U.S.A.) through the courtesy of Dr. M. Grogl. All other reagents were analytical grade.

Determination of Ca^{2+} movements

Variations in free Ca^{2+} concentration were monitored by measuring the changes in the absorbance spectrum of Arsenazo III [9] using an SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm at 28 °C. No free radical formation from Arsenazo III occurred under the conditions used [10–12]. Each experiment was repeated at least three times, and the Figures show representative experiments. Concentrations of the ionic species and complexes at equilibrium were calculated by employing an iterative computer program [13] modified from that described by Fabiato & Fabiato [14], taking into account the dissociation constants reported by Schwarzenbach *et al.* [15].

Estimation of mitochondrial membrane potential

Estimation of mitochondrial membrane potential *in situ* was done spectrophotometrically using the indicating dye safranine O at 28 °C [6,7]. *L. donovani* promastigotes were incubated in a medium containing 125 mM-sucrose, 65 mM-KCl, 10 mM-Tris/HCl, pH 7.2, 1 mM-$MgCl_2$ and 20 μM-digitonin. Absorbance changes were monitored on an SLM Aminco DW2000 spectrophotometer at the wavelength pair 511–533 nm [6,7]. Each experiment was repeated at least three times, and the Figures show representative experiments.

RESULTS

Characterization of the Ca^{2+}-transporting activities of permeabilized *L. donovani* promastigotes

When the promastigotes were added to a reaction medium containing MgATP, succinate, 3.5 μM free Ca^{2+} and digitonin to permeabilize the plasma membrane, a rapid decrease in Ca^{2+} concentration (3.2 nmol/min per mg of protein) started after a period of about 1 min and continued until a steady state was attained at a free Ca^{2+} concentration in the range 50–100 nM (Fig. 1, trace A). This Ca^{2+} concentration compares favourably with that detected in the cytosol of the intact promastigotes, as measured with fura-2 [5]. The subsequent addition of FCCP was followed by a large increase in medium Ca^{2+}, indicating the existence of an important mitochondrial Ca^{2+}-transporting activity. Subsequent addition of the Ca^{2+} ionophore A23187 resulted in the additional release of Ca^{2+} from an extramitochondrial pool. When the cells were added to the same reaction medium containing 1 μM-FCCP (Fig. 1, trace B), the rate of Ca^{2+} uptake by the permeabilized cells was much slower (0.8 nmol/min per mg of protein). This FCCP-insensitive Ca^{2+} uptake was completely inhibited by 500 μM-vanadate (Fig. 1, trace D). Therefore, when the cells were added to the reaction medium containing 500 μM-vanadate instead of FCCP (Fig. 1, trace C), only the mitochondrial Ca^{2+}-sequestering activity was observed. This activity was totally inhibited by the addition of 10 μM-Ruthenium Red (Fig. 1, trace E) and was much faster (2.4 nmol/min per mg of protein) than the non-mitochondrial one, but only decreased the medium Ca^{2+} concentration to about 0.7 μM. The broken line in Fig. 1, trace C shows that a further

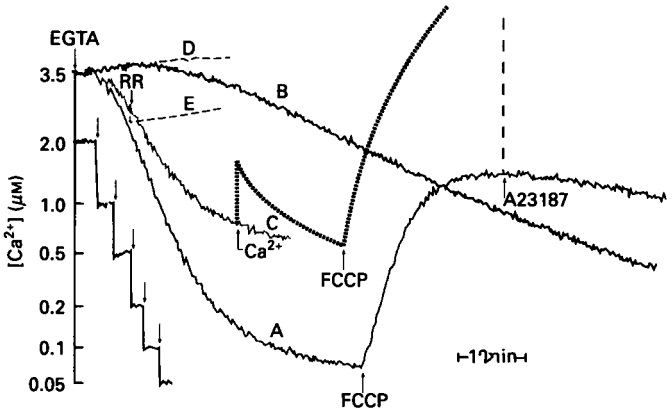

Fig. 1. Ca^{2+} uptake by digitonin-permeabilized *L. donovani*

The reaction medium contained 125 mM-sucrose, 65 mM-KCl, 10 mM-Hepes buffer, pH 7.2, 1 mM-$MgCl_2$, 1 mM-ATP, 2.5 mM-potassium phosphate, 2.0 mM-succinate, 3.5 μM-Ca^{2+}, 20 μM-digitonin and 40 μM-Arsenazo III in a total volume of 2.5 ml. Cells (0.76 mg of protein/ml) were added to this medium alone (trace A), or medium containing 1 μM-FCCP (trace B), 500 μM-sodium vanadate (trace C), 500 μM-sodium vanadate plus 1 μM-FCCP (trace D), or 500 μM-sodium vanadate plus 10 μM-Ruthenium Red (RR) (trace E). The calibration was performed by the sequential addition of known concentrations of EGTA. FCCP (1 μM) or Ca^{2+} ionophore A23187 (10 μM) was added where indicated. For trace C, 6.25 nmol of $CaCl_2$ was added where indicated. Rates of Ca^{2+} uptake in nmol/min per mg of protein: A, 3.2; B, 0.8; C, 2.4.

addition of 6.25 nmol of $CaCl_2$ was followed by a new decrease in medium Ca^{2+} until the previous set point was again reached, and that the subsequent addition of FCCP was followed by the release of all accumulated Ca^{2+}. This indicates the low affinity of the mitochondrial Ca^{2+} transport system, rather than its saturation with Ca^{2+}. Similar results were obtained using higher initial Ca^{2+} concentrations (Fig. 2*a*). These results suggest that the non-mitochondrial Ca^{2+}-transporting activity, probably represented by the endoplasmic reticulum, is responsible for the Ca^{2+}-buffering activity at concentrations below 0.7 μM.

The following experiments were designed to characterize further the properties of Ca^{2+} transport by *L. donovani* mitochondria *in situ*. These experiments were performed in a reaction medium containing 8 μM-Ca^{2+} in the absence of ATP, to prevent Ca^{2+} accumulation by the non-mitochondrial Ca^{2+} pool. The addition of digitonin was followed by a decrease in the medium free Ca^{2+} concentration at a level similar to that observed in the experiment of Fig. 1, trace C (about 0.7 μM, vanadate present) (Fig. 2*a*). This indicates that the presence of ATP does not affect Ca^{2+} transport by mitochondria if succinate is present. A further addition of 25 nmol of Ca^{2+} was followed by a further decrease in medium Ca^{2+}, until a steady state was reached at the Ca^{2+} concentration which preceded the Ca^{2+} addition. The experiment in Fig. 2(*b*) shows that the accumulation of Ca^{2+} was limited by the absence of phosphate, but was completely restored by the inclusion of 1 mM-phosphate. The addition of Ruthenium Red was followed by a low rate of net Ca^{2+} efflux, indicating that, as in vertebrate mitochondria [3], this steady state is characterized by a low rate of Ca^{2+} cycling across the mitochondrial inner membrane.

Estimation of the mitochondrial membrane potential of *L. donovani in situ*

We have shown that, contrary to the proposition [16] that only vertebrate mitochondria possess the electrophoretical Ca^{2+} uniporter, *T. cruzi* epimastigotes [1,2,17] and other protozoa [18]

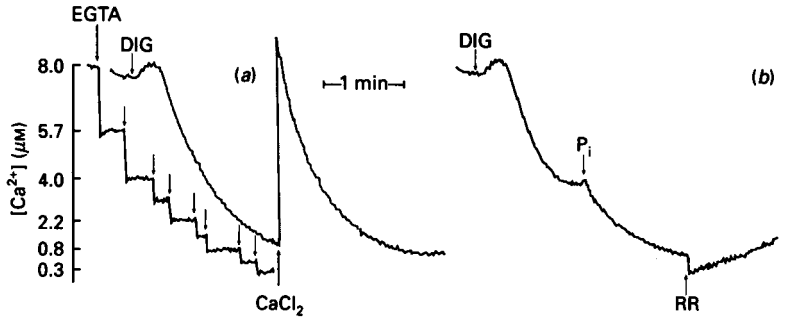

Fig. 2. Ca^{2+} transport by *L. donovani* mitochondria *in situ*

The reaction medium was similar to that described in Fig. 1, with the exception that ATP was absent in (*a*) and (*b*) and phosphate was absent in (*b*). The following additions were made where indicated: 20 μM-digitonin (DIG), 25 nmol of $CaCl_2$ (*a*), 1 mM-potassium phosphate (P_i) and 5 μM-Ruthenium Red (RR; *b*).

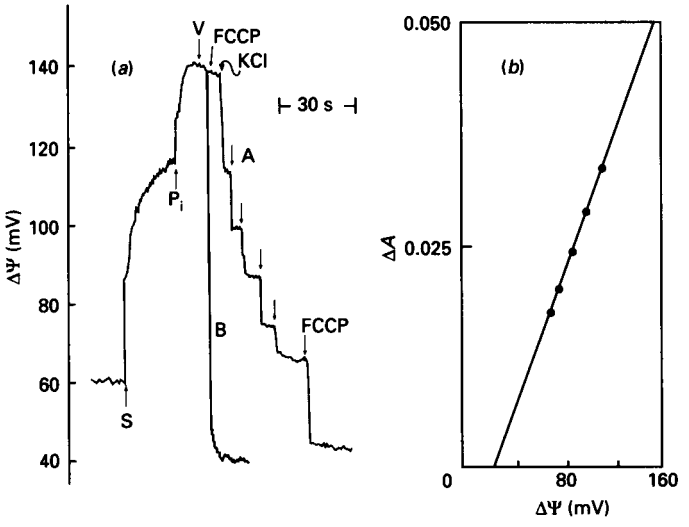

Fig. 3. Determination of the membrane potential of *L. donovani* mitochondria *in situ*

The cells (0.79 mg of protein/ml) were added to a potassium-free medium containing 200 mM-sucrose, 10 mM-Na-Hepes buffer, pH 7.0, 2.0 mM-sodium succinate, 1.0 mM-$MgCl_2$, 1.0 mM-EGTA and 20 μM-digitonin in a total volume of 2.5 ml. Trace A: safranine (S, 10 μM), 2.0 mM-sodium phosphate (P_i) and 0.2 μg of valinomycin/ml (V) were added where indicated. A titration of ΔΨ was obtained by the sequential addition of KCl to give the final concentrations of 1.3, 2.6, 3.9, 6.5 and 8.1 mM respectively. The ΔΨ value after each KCl addition was determined using the Nernst equation [6] as shown in (*b*). Trace B, safranine (S, 10 μM), 2.0 mM-sodium phosphate (P_i) and 1 μM-FCCP were added where indicated.

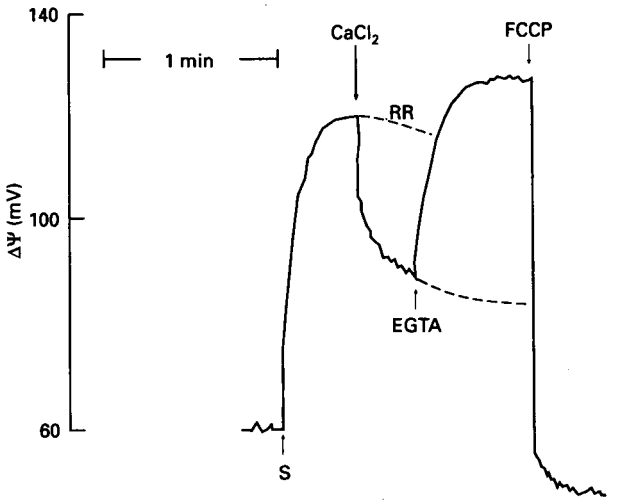

Fig. 4. Changes in the mitochondrial ΔΨ of *L. donovani* promastigotes associated with Ca^{2+} accumulation by mitochondria *in situ*

The experimental conditions were similar to those of Fig. 3, except that the concentration of EGTA was 80 μM, and 2.0 mM-sodium phosphate was present from the beginning. $CaCl_2$ (100 μM), EGTA (1 mM) and FCCP (1 μM) were added where indicated. RR indicates that 10 μM-Ruthenium Red was present in the medium from the beginning.

also possess such a mitochondrial mechanism for Ca^{2+} transport. Initially we used the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the inner membrane to estimate the changes in mitochondrial membrane potential (ΔΨ) associated with Ca^{2+} transport by these cells [17]. Since the quantification of ΔΨ by this method requires the accurate determination of mitochondrial volume and of the mitochondrial protein concentration (per mg of cellular protein), we have adapted the safranine technique [6] to measure the ΔΨ of mitochondria *in situ* in permeabilized cells [7]. This technique has permitted a good estimation of ΔΨ in *T. cruzi* epimastigotes as well as allowing us to monitor changes caused by cation transport, metabolic inhibitors and drugs [2,7].

The addition of 10 μM-safranine to a reaction medium containing digitonin-permeabilized *L. donovani* promastigotes was followed by an increase in absorbance at the wavelength pair 511–533 nm (Fig. 3) compatible with the stacking of the dye to the energized mitochondrial inner membrane [6,7]. In agreement with the behaviour of *T. cruzi* epimastigotes [7] and vertebrate mitochondria [6], the addition of P_i caused an increase in ΔΨ of about 20 mV, compatible with a decrease in the chemical component (pH gradient; ΔpH) of the electrochemical proton gradient ($\Delta\mu H^+$) due to the influx of phosphate through the P_i/H^+ symporter [6]. The observed increase of the absorbance at 511–533 nm was completely reversed by the addition of the protonophore uncoupler FCCP, confirming that the change in absorbance was in fact a function of the membrane potential. Fig. 3 also shows a titration of ΔΨ by the sequential addition of known concentrations of KCl after valinomycin was included in the medium to permit the electrophoretical uptake of K^+. The magnitude of this membrane potential could be estimated as being of the order of 140 mV on the basis of the extent of the safranine shift and in comparison with a calibration curve where ΔΨ values were calculated using the Nernst equation [6]. We took advantage of this technique to ascertain whether the

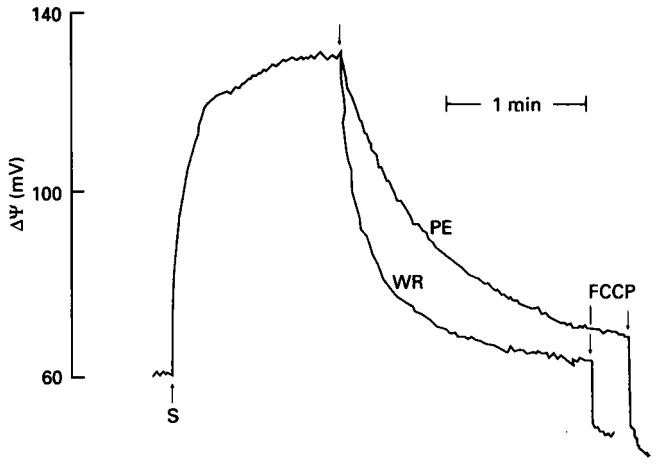

Fig. 5. Effect of pentamidine and WR-6056 on the mitochondrial membrane potential of *L. donovani in situ*

The experimental conditions were exactly the same as those in Fig. 4, except that the EGTA concentration was 1 mM. Pentamidine (PE, 300 μM), WR-6026 (WR, 200 μM) and FCCP (1 μM) were added where indicated.

transport of Ca^{2+} by mitochondria is associated with changes in $\Delta\Psi$, and whether the mitochondria could be a target of the lipophilic cationic compounds pentamidine and WR-6026.

Fig. 4 shows that an important decrease in $\Delta\Psi$ was caused by addition of $CaCl_2$, which brought the free Ca^{2+} concentration to about 20 μM. We used this Ca^{2+} concentration in this experiment because EGTA was present from the beginning to chelate the contaminating Ca^{2+} in the medium and to permit the detection of the effect of exogenous Ca^{2+} addition on the membrane potential. This decrease in $\Delta\Psi$ was completely reversed when the medium free Ca^{2+} concentration was lowered to $< 10^{-8}$ M by the addition of excess EGTA. This was required because under these experimental conditions mitochondria were subjected to a high Ca^{2+} concentration and could not regenerate $\Delta\Psi$ due to the damaging effect of Ca^{2+} overloading (broken line). The subsequent addition of FCCP showed that mitochondria were still intact under these conditions and confirmed the electrophoretical nature of the Ca^{2+} influx into these mitochondria, since this decrease in $\Delta\Psi$ was prevented by the presence of Ruthenium Red. Fig. 5 shows that both WR-6026 (200 μM) and pentamidine (300 μM) caused a rapid and extensive decrease in $\Delta\Psi$ which was completed in both cases by the further addition of FCCP.

DISCUSSION

The present work extends our knowledge of the operation of intracellular Ca^{2+} pools in permeabilized *L. donovani* promastigotes. When these permeabilized cells were incubated in medium containing MgATP and respiratory substrates, they lowered the extracellular Ca^{2+} concentration to a submicromolar level (0.1 μM). This concentration compares favourably with that detected in intact promastigotes by Philosoph & Zilberstein [5] using fura-2. As has been observed with other protozoa [1,2,7,18,18a], and similar to the case in a variety of eukaryotic cells [3], intracellular Ca^{2+} uptake at high levels of free Ca^{2+} (> 1 μM) is probably mediated by the electrogenic mitochondrial uniporter, with a set point close to 0.7 μM. However, as the free Ca^{2+} concentration is lowered from 1 μM, eventually all uptake is probably due to the ATP-dependent Ca^{2+} sequestration by the endoplasmic reticulum. This suggests that the mitochondria of *L. donovani* promastigotes, as occurs with the mitochondria of other eukaryotic cells thus far investigated [3], and contrary to previous suggestions [5], are not involved in the regulation of the cytosolic Ca^{2+} concentration under normal physiological conditions. In addition, we have established the experimental conditions for accurate quantitative determinations of the mitochondrial membrane potential of digitonin-permeabilized *L. donovani* promastigotes *in situ*. Our finding of a membrane potential of about 60 mV in uncoupled mitochondria *in situ* (Figs. 3–5) is in excellent agreement with the data of Åkerman & Wikstrom [6]. Under these conditions the membrane potential is counteracted by a pH gradient (acid inside), resulting in zero electrochemical proton gradient [19]. These results indicate that these mitochondria, despite some peculiar characteristics (such as their large size, the existence of only one mitochondrion per cell, RNA-editing, etc.) behave similarly to most vertebrate mitochondria with regard to their electrochemical proton gradient. In addition, we have demonstrated that two potent anti-leishmanial drugs, pentamidine and WR-6026, are able to collapse this mitochondrial membrane potential.

Pentamidine has been used for the treatment of visceral leishmaniasis for over 30 years, although its mode of action has not been completely elucidated. Ultrastructural studies of *L. tropica* isolated from a patient before and after therapy with pentamidine have shown morphological changes in the kinetoplast and mitochondria, with disintegration of the kinetoplast into a filamentous network and mitochondrial enlargement with the disappearance of cristae [20]. Ultrastructural studies of *L. mexicana amazonensis* exposed *in vitro* to pentamidine have also shown extensive mitochondrial disruption, with membrane and cristae fragmentation [21]. Our results are in agreement with these studies [20,21] and indicate that the mitochondria of *Leishmania* spp. are an important target for this drug.

WR-6026 is an 8-aminoquinoline with marked activity against *L. donovani* which is presently undergoing clinical trials against visceral leishmaniasis [22] and whose mechanism of action is also unknown. The property that pentamidine and WR-6026 have in common is that they are both lipophilic cationic drugs. Cationic drugs can be concentrated across membrane potentials into mitochondria [23]. Because of their positive charge they move into mitochondria through a mechanism driven electrophoretically by the inside-negative membrane potential. *In vitro* uncoupling of mitochondrial oxidative phosphorylation has been observed with several cationic drugs such as ethidium bromide [24,25], Nile Blue, Acridine Orange, pyronin Y, coriphosphine [26] and Crystal Violet [27]. Several of these compounds, such as ethidium bromide and Crystal Violet, are well-known leishmanicidal agents [28]. Our working hypothesis is that due to their cationic nature, pentamidine and WR-6026 are taken up by *L. donovani* mitochondria through an electrophoretic mechanism and then affect the mitochondrial membranes, causing uncoupling of oxidative phosphorylation in these parasites.

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