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Temperature-induced rapid increase in cytoplasmic free Ca^{2+} in pathogenic *Leishmania donovani* promastigotes

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Abstract **We demonstrate that the cytoplasmic free Ca^{2+} concentration in three virulent *Leishmania donovani* promastigote strains is maintained at 20–30 nM level whereas the avirulent promastigotes maintain $[Ca^{2+}]_i$ at 80–100 nM. Rapid exposure of virulent promastigotes to higher physiological temperature increases $[Ca^{2+}]_i$ many-fold. Use of $CaCl_2$, EGTA and Mn^{2+} confirmed that both release of Ca^{2+} from internal pool(s) and influx from outside milieu are responsible for this increase in $[Ca^{2+}]_i$. Application of oligomycin, CN^-, and nigericin indicated the non-mitochondrial pH_i sensitive pool as the possible intracellular origin for internal Ca^{2+} release. Exposure of these cells to acidic environment had no influence on $[Ca^{2+}]_i$. In contrast, the avirulent promastigotes or freshly isolated amastigotes had shown no effect of heat-shock or pH shock on $[Ca^{2+}]_i$ of these cells. Our results indicate that the upward shift in temperature may play a role in signal transduction events in morphogenetic transformation of *L. donovani* promastigotes that involves mobilization of Ca^{2+} in cytoplasm.**

Key words: Heat shock $[Ca^{2+}]_i$; *Leishmania donovani*

1. Introduction

The human protozoan pathogen *L. donovani* is the causative agent for Kala-azar or visceral leishmaniasis that is a major health hazard in many parts of the tropical world including India [1]. The parasite has a strictly digenic life cycle. Following bite by sandfly, the highly motile flagellated promastigote or the vector form is internalized in liver macrophages. Here the organism rapidly undergoes a morphogenetic transformation into the aflagellated amastigote form in the phagolysosomal complex of macrophages. The organism overcomes the host defence mechanism, multiplies and invades neighbouring macrophages that lead to pathogenicity [2]. During this pathogenic transformation, the organism has to adopt to a shift in temperature from 22–24°C in the vector to 37°C in the mammalian host. It is likely that along with other, as yet, unknown biochemical signals, the upward shift in temperature acts as a signal in facilitating this morphogenetic transformation. In fact, controlled adaptation of *L. braziliensis* promastigotes to higher culture temperature can convert the promastigote to amastigote-like forms which by several biological and biochemical criteria appear to behave like true amastigotes [3,4]. Certain heat-shock proteins are also found to appear when the parasite is exposed to higher temperature [5,6].

Calcium ion is now recognized as a major intracellular signal through which external stimuli evoke a variety of short-term and long-term responses in eucaryotic cells [7–9]. Although, the physiological role of Ca^{2+} as a second messenger in *Leishmania* spp. has not yet been established, evidence is rapidly accumulating to suggest the presence of an elaborate machinery for maintaining Ca^{2+} homeostasis in the cytoplasm. A plasma membrane Ca^{2+}-ATPase, acting as a putative Ca^{2+} extrusion pump have been characterized in our laboratory [10–12]. As in case of higher eucaryotes, the kinetoplastida group of organisms also contain at least two intracellular organelles that can store Ca^{2+}. More specifically, using digitonin permeabilized cells the presence of a mitochondrial pool and a pH-sensitive pool has been demonstrated in *Leishmania* spp. [13–16]. In vitro morphogenetic transformation and flagellar movement are also severely affected in presence of Ca^{2+} channel blockers and calmodulin antagonists [17].

We report here that the virulent *L. donovani* promastigotes that we tested have cytosolic free Ca^{2+} concentration or $[Ca^{2+}]_i$ at 20–30 nM which is significantly lower than in case of non-virulent strains or higher eucaryotes. More importantly, when virulent strains are exposed to higher temperature, it results in a rapid rise of cytoplasmic free Ca^{2+} concentration that is essentially released from the internal non-mitochondrial pool.

2. Materials and methods

2.1. Strains

All the *Leishmania* strains used for this work are clinical isolates from confirmed Kala-azar patients [18]. Virulent and pathogenic *Leishmania* strains slowly loose their virulence on repeated subculturing in a suitable liquid medium, e.g. medium 199. On the other hand, if the freshly isolated pathogens are maintained and transferred whenever necessary (or every 3–5 months) in golden hamster as the experimental model, the virulence of the organism remains unaffected. Accordingly, AG83 and GE1 were available both in their virulent (V) and non- virulent (NV) forms. AG83 (NV) and GE1 (NV) were gifts from Dr. S. Duttagupta of our Institute. The strainUR6 has now been renamed as MHOM/IN/1978/UR-6 [19]. This strain is grown and maintained only in solid blood agar medium [10] and has totally lost its virulence.

2.2. Media

Liquid medium 199 supplemented with 20 mM HEPES buffer, pH 7.4, 20% fetal calf serum, 100 units of penicillin and 100 μg of streptomycin was used for transformation of amastigotes into promastigotes. Promastigote cells were subculture in liquid medium 199 supplemented with 20 mM HEPES buffer, pH 7.4, 10% fetal calf serum and same amount of penicillin-streptomycin. Virulent AG83 (V) and GE1 (V) were rendered non-virulent after 20–25 times of subculturing in this liquid medium 199.

2.3. Test for virulence

The virulence is tested by injecting 1×10^7 promastigotes into the heart of golden hamsters. Spleen of two months post-infection were excised and parasite load were seen by usual procedures. The absence of parasite in the spleen indicated non-virulent status of the parasite strain.

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2.4. Isolation of amastigotes

Amastigotes were isolated and purified from infected spleens of golden hamsters by a modified method of Meade et al. [20].

2.5. Chemicals

Nigericin, oligomycin, EGTA, Fura-2/AM, calcium ionophore 4BrA23187, HEPES, Triton X-100, $CaCl_2$ and medium 199 were all purchased from Sigma Chemical Co. Penicilin-streptomycin, fetal calf serum were purchased from Gibco. All other reagents were of analytical grade.

2.6. Spectrofluorimetric methods

Fura-2 determinations were performed essentially as described by Philosoph and Zilberstein [13]. After harvesting, the cells were washed twice at 6000 rpm for 10 min at 4°C in buffer A which contained 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. Cells were resuspended to a final density of 1×10^9 cells/ml in loading buffer that contained 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, 1.5% sucrose and 50 mM HEPES at pH 7.4, and 6 μM Fura-2/AM. The suspension was incubated for 1 h in a 37°C waterbath with occasional agitation. Subsequently, the cells were washed four times with ice-cold buffer A to remove extracellular dye. Cells were resuspended to a final density of 1×10^9 cells/ml in buffer A and were kept at 24°C room temperature. For fluorescence measurements, a 125 μl aliquot of the cell suspension was diluted into 2.4 ml of buffer A. Fura-2 was excited at 340 nm wavelength and emitted fluorescence at 510 nm wavelength was passsed through a 10 nm bandpass filter of Hitachi 4010 Spectrofluorimeter. The measurement of $[Ca^{2+}]_i$ were done at 30°C. For the calculation of $[Ca^{2+}]_i$, the fluorescence signal obtained from whole cell (F_1), after addition of 1 mM EGTA (F_2), followed by lysing the cells with 0.04% Triton X-100 in presence of 30 mM Trizma-base (F_3) and then 4 mM $CaCl_2$ (F_4) respectively were used [21]. The formula is:

$$[Ca^{2+}]_i = K_d \times \frac{(F_2 - F_3)}{(F_4 - F_1)} \text{ nM.}$$

K_d value of 224 nM was used [22].

Heat-shock experiments were done by shifting the cuvette containing 125 μl of original cell suspension in 2.4 ml buffer A at pH 7.0 from 24°C to the thermostatically controlled cuvette holder of the fluorimeter at the indicated temperature. The monitoring of the fluorescence signals were started immediately after the cuvette in the cuvette holder of the instrument. It was observed that the temperature of the cell suspension attained the holder temperature within 30 sec. of exposure to heat.

3. Results and discussion

3.1. Cytoplasmic free Ca^{2+} or $[Ca^{2+}]_i$ in virulent strains

Measurement of cytoplasmic free Ca^{2+} concentration or $[Ca^{2+}]_i$ by loading with Fura-2/AM showed significant differences between virulent and non-virulent strains. Fig. 1 shows that the cytoplasmic Fura-2 fluorescence signal from AG83 (V) is significantly closer to the minimum fluorescence of total Fura-2 than what is observed with AG83 (NV). When these measurements were extended to other available strains, similar differences were observed. These results are summarized in Table 1. Clearly, the pathogenic promastigotes AG83 (V) and GE1 (V) maintain $[Ca^{2+}]_i$ at the level of 20 nM which is significantly lower than the typical cytoplasmic free Ca^{2+} concentration of 100 nM in higher eucaryotes. Interestingly, the non-pathogenic forms of these two strains as also the non-pathogenic UR-6 strain all show $[Ca^{2+}]_i$ of 80–100 nM that compares well with the measured values of higher eucaryotes. We also noted that the amastigote form of AG83 (V) also has $[Ca^{2+}]_i$ of 80 nM, close to the value reported for higher eucaryotes. The promastigote form of this virulent pathogen thus has a much lower $[Ca^{2+}]_i$ than is observed in other eucaryotic cell types. Similar low values for $[Ca^{2+}]_i$ were earlier reported for only the blood-stream form of *Trypaosoma brucei* [23] and the epiamastigote form of *T. cruzi* [24]. Philosoph and Zilberstein had earlier reported a higher value (80–100 nM) for a strain of *L. donovani*, the virulence status of which was, however, not stated [13]. The promastigote form of the virulent pathogen thus has substantially lower levels of $[Ca^{2+}]_i$ that probably reflects some physiological significance the full implication of which remains to be elucidated.

3.2. Increase in $[Ca^{2+}]_i$ on exposure to heat

When the virulent promastigotes were exposed to heat that led to rapid upward shift of medium temperature from 24°C to 34°C, a dramatic increase in $[Ca^{2+}]_i$ could be observed. Fig. 2 shows the results of a typical set of experiments that were repeated 3–5 times. Heat shock of GE1 (V) strain in presence of exogenous Ca^{2+} (1 mM) resulted in a rapid rise of $[Ca^{2+}]_i$

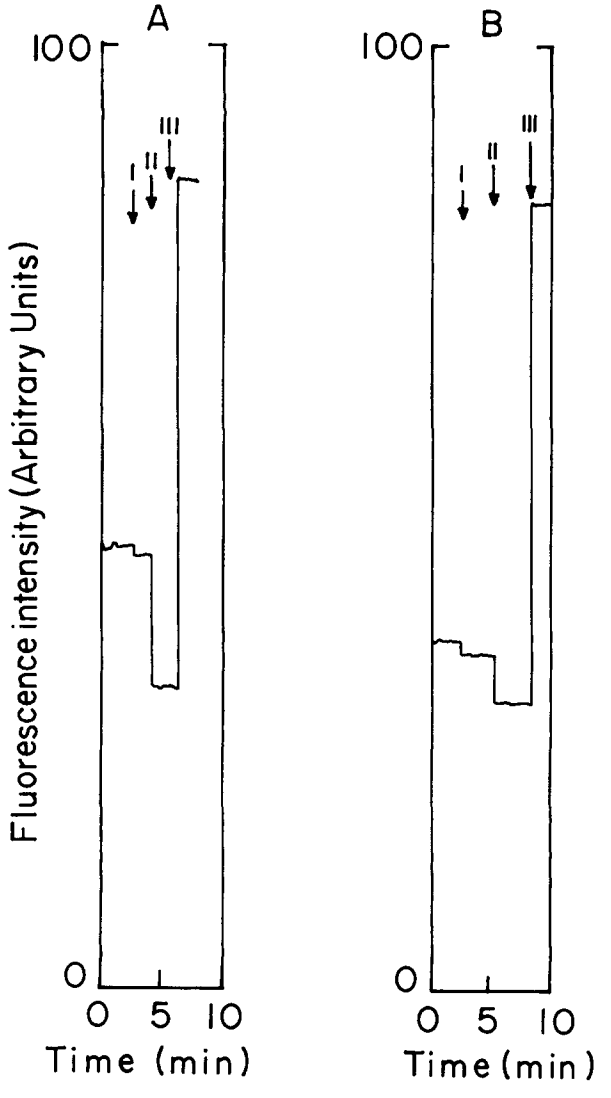

Fig. 1. Measurement of $[Ca^{2+}]_i$ in *Leishmania donovani* promastigotes. Cells were loaded with Fura-2/AM as described in section 2 and suspended in buffer A. Fluorograms showing the relative fluorescence signal of Fura-2 loaded (A) Nonvirulent and (B) virulent cells after treatment with 1 mM EGTA (↓I), 30 mM Trizma base and 0.04% Triton X-100 (↓II) and 4 mM $CaCl_2$ (↓III).

from 20 nM to 400 nM that slowly stabilized to 100 nM within 10 min (curve a). In a separate experiment, when all exogenous contaminating Ca^{2+} was chelated with EGTA (1 mM), one could still demonstrate a rapid rise of $[Ca^{2+}]_i$ to 270 nM (curve b) indicating clearly the involvement of one or both the internal pools in this rise of $[Ca^{2+}]_i$. Curve c shows a similar response to upward shift of temperature by AG83 (V). Further, addition of Mn^{2+}, a surrogate for Ca^{2+} that shares the same channel results in rapid quenching of Fura-2 signal. Association of Mn^{2+} with Fura-2 is known to quench the fluorescence signal. In sharp contrast, non-pathogenic AG83 (NV), GE1 (NV) and UR-6 did not exhibit any such effect of heat-shock on $[Ca^{2+}]_i$ (curves d, e and f). Interestingly, the amastigote form of AG83 also failed to respond to the heat-shock (data not shown). It appears that the rise in cytoplasmic free Ca^{2+}, due to its release from internal pool(s) on upward shift of temperature, is a property very characteristic of virulent strains of *L. donovani* that presumably are destined to undergo morphogenetic transformation in host macrophages. This effect of heat-shock is maximum when the cells are exposed to a rapid temperature shift from 24 to 34°C. In fact, rapid shift of temperature until 30°C failed to demonstrate any significant rise in $[Ca^{2+}]_i$. The viability of the cells in all cases after exposure to heat were checked and was found to remain unaffected.

3.3. Release of Ca^{2+} is non-mitochondrial in origin

When oligomycin (0.8 μg/ml) and NaCN (1 mM) were added immediately after 1 min of giving heat-shock, neither $[Ca^{2+}]_i$ of resting cells (Fig. 3A (b)) nor the release of Ca^{2+} after heat-shock (Fig. 3A (a)) were affected. In other trypanosomatide, employing permeabilized cells, it was shown earlier that addition of electron transport chain inhibitors prevented uptake of Ca^{2+} in mitochondria but did not result in any release of Ca^{2+} from the mitochondrial pool [13–15,25]. When Mn^{2+} (100 μM) was added to the cells that were first exposed to upward shift of temperature and then treated with the inhibitors (Fig. 3A (a)), a rapid quenching of Fura-2 signal could be observed. Presumably, upward shift of temperature opened the Ca^{2+} channel and allowed rapid influx of Mn^{2+} to displace Ca^{2+} from Fura-2-Ca^{2+} complex in the cytoplasm.

As nigericin is known to release Ca^{2+} from a non-mitochondrial pool in intact cells [16], we applied nigericin (2.7 μM) at the moment after heat-shock when heat sensitive Ca^{2+} pool is supposed to become emptied (Fig. 3B (a)). As no additive effect was observed it can be concluded that this heat-senstitive pool and pH_i-sensitive pool is overlapping. As a control when nigericin (2.7 μM) was added at 24°C, it immediately increased the

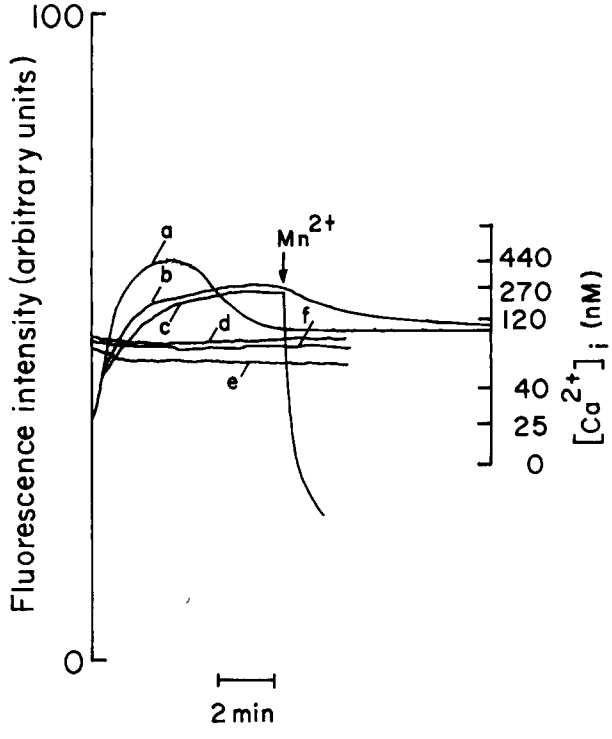

Fig. 2. Monitoring of heat-sensitivity of $[Ca^{2+}]_i$ by Fura-2/AM loaded *Leishmania donovani* promastigotes. After Fura-2 loading cells were suspended in buffer A pH 7.0 at 24°C room temperature as described in section 2. Fluorogram indicated, GE1 (V) promastigotes shifted to 34°C in presence of 1 mM $CaCl_2$ (curve a), 1 mM EGTA (curve b), AG83 (V) promastigotes shifted to 34°C and $MnCl_2$ (100 μM) added at the indicated times (curve c). UR6 (curve d), AG82 (NV) (curve e) and GE1 (NV) (curve f) shifted to 34°C as above.

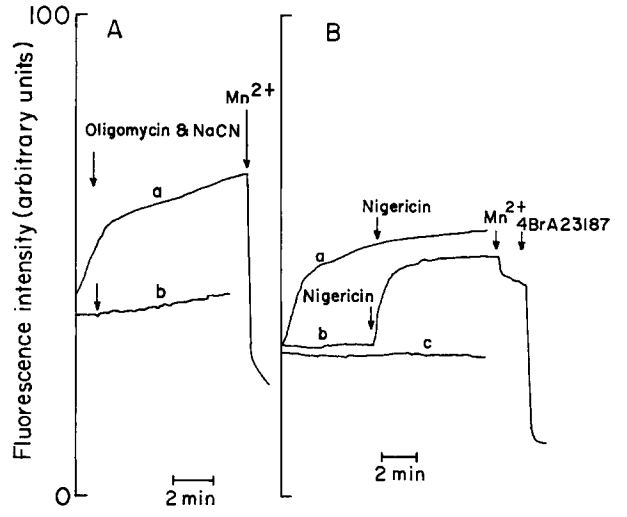

Fig. 3. The effect of mitochondrial inhibitors and pH change on heat-sensitive Ca^{2+} release. AG83 (V) promastigotes were loaded with Fura-2/AM as described in section 2. (A) Fura-2/AM loaded cells were exposed to heat-shock from 24°C to 34°C and immediately after 1 min oligomycin (0.8 μg/ml) and NaCN (1 mM) were added simultaneously. $MnCl_2$ (100 μM) added at indicated time (curve a). The control experiment was done without shift in temperature only (curve b) (B) Fluorescence signals from cells were monitored after heat-shock and nigericin (2.7 μM) was added at the indicated times (curve a), at 24°C temperature nigericin (2.7 μM), $MnCl_2$ (100 μM) and calcium ionophore 4BA23187 (5 μM) were included (curve b). Cells were shifted from buffer at pH 7.0 to pH 5.0 (curve c).

Table 1
The measured values of $[Ca^{2+}]_i$ in different strains of *Leishmania donovani* (average of five determinants)

Name of the strain	Morphogenetic form	Status of the strain	Measured value of $[Ca^{2+}]_i$ in nM
(1) AG83 (V)	Promastigote	Pathogenic	20
(2) GE1 (V)	Promastigote	Pathogenic	18
(3) AG83 (NV)	Promastigote	Nonpathogenic	80
(4) GE1 (NV)	Promastigote	Nonpathogenic	66
(5) UR-6	Promastigote	Nonpathogenic	100
(6) AG 83	Amastigote	Pathogenic	85

$[Ca^{2+}]_i$ in virulent AG83 (V) cells (Fig. 3B (b)). Interestingly, Mn^{2+} quenching after nigericin treatment does not show any dramatic effect at this stage. This indicates that the increased level of $[Ca^{2+}]_i$ released from the internal pool in absence of upward temperature shift is unable to induce rapid influx of Ca^{2+} from outside milieu. Taken together these results indicate that the release of Ca^{2+} from intracellular pool and rapid influx of Ca^{2+} are two separate phenomena occurring simultaneously. We also noted that subjecting *L. donovani* promastigotes, both virulent and non-virulent types to shift towards acidid pH, the presumed pH in phagolysosomal complex, (from pH 7.0 to 5.0) did not have any detectable influence on $[Ca^{2+}]_i$ (Fig. 3B (c)). This result indicates that pH shock does not act as stimulus in the morphogenetic transformation of promastigotes, assuming $[Ca^{2+}]_i$ has a role to play in the process. Upward temperature shift along with concomitant Ca^{2+} release from the internal non-mitochondrial pool may, however, be a significant initial event in morphogenetic transformation of the parasite. Detailed characterization with permeabilized cells is in progress to demonstrate the nature of Ca^{2+} release from the intracellular pool.

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