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Possible Involvement of Ca^{2+} in the *in vitro* Morphogenetic Transformation of *Leishmania donovani* Amastigotes to Promastigotes

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Growth of *L. donovani* promastigote cells is unaffected by EGTA and nifedipine. Calcium ionophore A23187, trifluoperazine and calmidazolium have profound inhibitory effects at 1μM concentrations. Trifluoperazine and other calmodulin antagonists blocked the respiration and motility of promastigote cells only at much higher concentrations (30μM). Nifedipine and Quin 2-AM in contrast to their lack of effect on growth of promastigote cells show strong inhibition in the transformation of amastigote to promastigote form at low concentrations of 0.5μM and 2μM respectively. Calmodulin antagonists and calcium ionophore affect the transformation process at their respective growth inhibitory concentrations. These observations suggest that extracellular calcium ion is probably not obligatory for the promastigote cell division but has a definite role in the morphogenetic transformation of amastigote to promastigote form.

Key Words: Chelators, Channel blockers, Calmodulin antagonist, Morphogenetic transformation

Introduction

The parasitic protozoan *Leishmania donovani* is the causative agent for kala-azar, a fatal disease that is endemic in eastern regions of India and threatens to become a major health hazard in near future (WHO 1987). The parasite has a strictly digenic life cycle. After infection due to a sandfly bite, the highly motile and strongly aerobic flagellated promastigote is rapidly internalized in liver macrophages. Here it dramatically and rapidly undergoes a morphogenetic transformation into the aflagelatted pathogenic amastigote form in the phagolysosomal complex of the macrophage. The organism overcomes the host defense mechanism, multiplies and invades neighbouring macrophages, leading to pathogenicity (Killick-Kendrick 1979). Cellular differentiation from the promastigote to amastigote form is most probably dependent on some as yet unidentified signal(s) from the host macrophage milieu. The biochemical nature of the signal, its transduction and amplification remains to be elucidated.

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Calcium ion is now recognized as a major intracellular signal through which external stimuli evoke a variety of cellular responses in eukaryotic cells. Movement or mobilization of Ca^{2+} leading to the transitory rise in its concentration at the site of action, its interaction with response elements and the subsequent rapid removal of excess free Ca^{2+} is a general feature of Ca^{2+} messenger system (Rasmussen & Goodman 1977, Carafoli 1987, Berridge Michael 1987). High-affinity, membrane inserted Ca^{2+}-pumps, voltage or ligand-gated Ca^{2+} channels, and Ca^{2+}-calmodulin-dependent protein phosphorylating systems are characteristics of Ca^{2+} messenger system. We have recently isolated from the plasma membrane of *L. donovani* promastigote a high-affinity Ca^{2+}-ATPase that spans the plasma membrane and has its catalytic site directed towards the cytoplasmic face (Ghosh et al. 1990). The orientation of the enzyme is suggestive of its role as an extrusion pump for Ca^{2+}. More interestingly, the enzyme is under regulatory control of endogenous calmodulin that has also been purified and characterized (Mazumder et al. manuscript under preparation). Work from other laboratories with membrane-permeable fluorescent Ca^{2+}-chelators (Philosoph & Zilberstein 1990) and with plasma membrane vesicles from *L. braziliensis* promastigotes (Benain & Romero 1990) are also indicative of Ca^{2+} movement across the plasma membrane into undefined internal organelles. Notwithstanding these findings so far, no definitive role for Ca^{2+} has been demonstrated in the physiology or in the life cycle of this pathogenic organism. In order to address such questions, we have now monitored the effect of a large number of Ca^{2+} related drugs or chemicals on the growth of the promastigote form of the organism and also on the amastigote to promastigote transformation in an *in vitro* system. Our preliminary results suggest that Ca^{2+} movement has a role in the morphogenetic transformation of the organism but not in cell division.

Materials and Methods

Reagents : Nifedipine, calmidazolium, calcium ionophore A23187, trifluoperazine, W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride)], Quin 2-AM, EGTA [Ethylene glycol bis (2-aminoethylether)-N,N,N'N-tetraacetic acid], CDTA (Trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid) were all purchased from Sigma Chemical Company. HEPES (N-2-hydroxyethylpiperazine-N'-d-ethansulfonic acid), Scheneider's medium, penicillin-streptomycin and fetal bovine serum were from GIBCO. Nifedipine, calmidazolium, W-7, trifluoperazine and Quin 2-AM were dissolved in dimenthylsulfoxide. Calcium ionophore A23187 was dissolved in a solution of ethanol and dimethyl sulfoxide mixed at a ratio of 1:3 (v/v).

Maintenance and preparation of L. donovani cells : The promastigote cell line used in our experiments was a clinical isolate from a kala-azar patient. The strain, originally designated UR6, has been renamed MHOM/IN/1979/UR-6 (Chakravorty & Das 1988). The strain has now become nonvirulant. We have used this cell line in our experiments because these cells can be grown and maintained easily on a blood-agar medium that has been described elsewhere (Ghosh et al. 1990). Amastigotes of *L. donovani* were also derived from a clinical isolate (Ghosh et al. 1987). This virulant strain is designated as AG83 and is maintained by serial passage in BALB/c mice. The organism was passaged by tail vein injection of freshly transformed 1×10^7 promastigote cells from splenic homogenates of infected mice. BALB/c mice were sacrificed 8 weeks after infection.

Amastigotes were isolated and purified from infected spleens by a described method (Meade et al. 1984).

For growth experiments promastigote cells were harvested during logarithmic phase of growth from blood agar medium, isolated by centrifugation at 1100 × g for 10 min and washed twice in phosphate buffered saline (PBS). These PBS washed cells were then inoculated in GC1 medium at a concentration of 1×10^6 ml^{-1} (Choudhury et al. 1982). Counts were taken at different time intervals by means of microscopic observation using a haemocytometer.

For transformation experiments 1×10^6 cells were inoculated in 1ml Schneider's medium (Hendricks et al. 1979) supplemented with 20 mM Hepes buffer *p*H 7.4, 30% fetal bovine serum and 100 µg of both penicillin and streptomycin in presence of drugs. After 2 hr of incubation the drugs were washed out and freshly incubated at 22°C by resuspending in drug free medium. Transformation was assessed after 45 hr of incubation by counting motile flagellated promastigote cells on haemocytometer under phase-contrast microscope. Results are expressed in percent transformation taking the control as 100 per cent.

Respiration studies were done polarigraphically by a clark type oxygen electrode (Yellow spring instruments, Model 5 6, USA).

Results

Effect of Ca^{2+}-chelators or Ca^{2+}-ionosphore on the growth of ***L. donovani*** *promastigote*

Parasitic protozoa with complex life cycles are difficult to grow *in vitro*. *Leishmania* spp. are no exception to this common experience. Methods have been established to grow several *Leishmania* spp. including *Leishmania donovani* in complex or defined media (Hendricks & Wright 1979, Steiger & Black 1980). The cultural form morphologically corresponds to the vector or the flagellated, motile promastigote form of the organism. The amastigote or aflagellated pathogenic form of the organism has not yet been successfully grown in culture. We tested the effect of Ca^{2+}-chelators or Ca^{2+}-ionophore A23187 (Pressman 1976) on the growth of the cultural or promastigote form of this organism. Figure 1 shows the result of such an experiment. Addition in the culture of EGTA, a specific high-affinity chelator of Ca^{2+} or CDTA, a chelator for both Ca^{2+} and Mg^{2+} (data not shown), had no effect on promastigote cell division. Clearly entry of extracellular Ca^{2+} is not obligatory for this cellular process. In contrast, addition of Ca^{2+} ionosphore in very low concentrations or of the membrane permeable Ca^{2+}-chelator Quin 2-AM (Tsien et al. 1982) at fairly high concentrations had significant effects on the

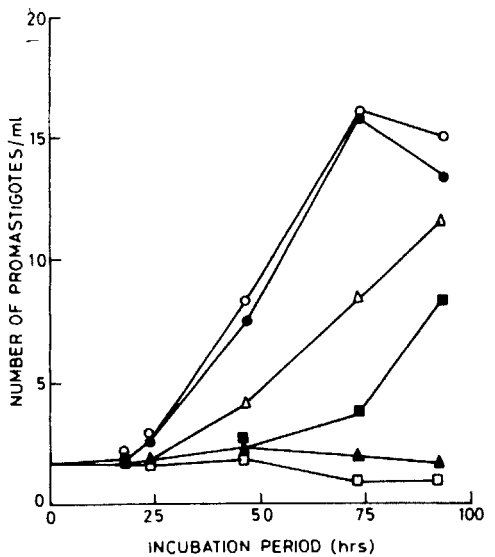

Figure 1 *L. donovani* promastigote cell growth in GC1 medium supplemented with 1 mg per 100 ml both of hemin and folic acid at 22°C in presence of calcium ion chelators and calcium ionophore A23187, —●— vehicle control (DMSO), —○— 1 mM EGTA, —△— 45 µM Quin 2-AM, —▲— 120 µM Quin 2-AM, —■— 1.0 µM calcium ionophore, —□— 2.0 µM calcium ionophore

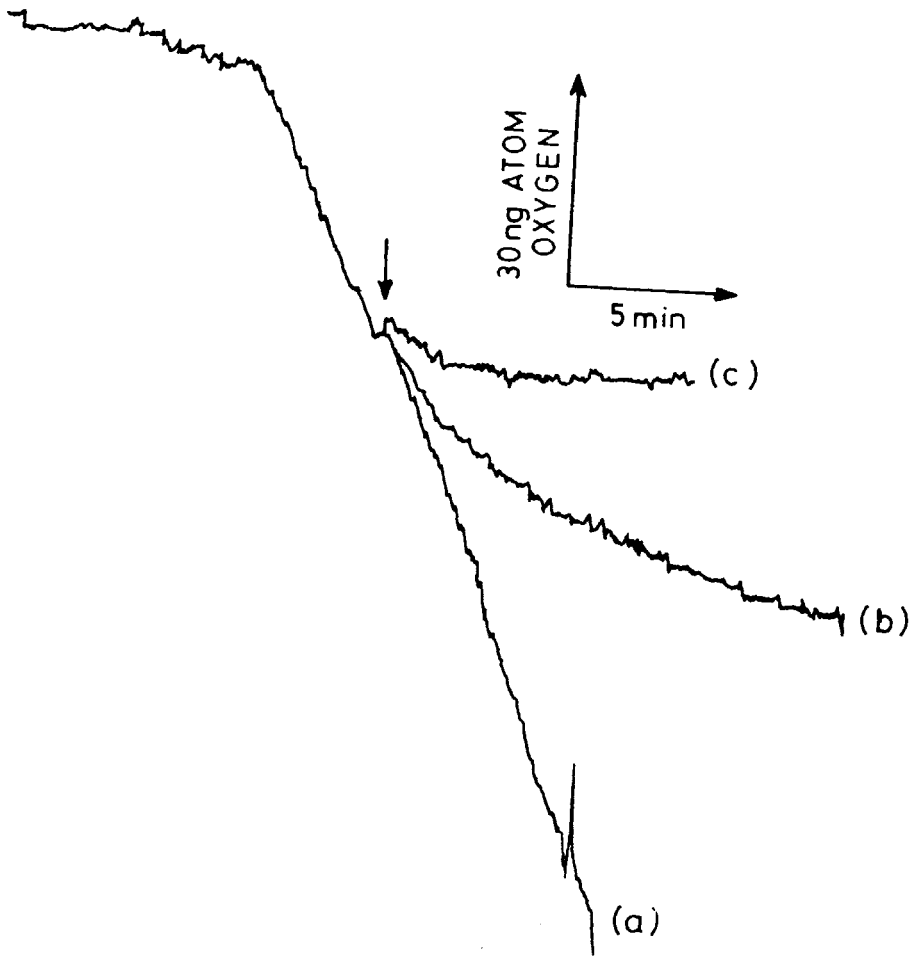

Figure 2 Inhibition of oxygen uptake by *L. donovani* promastigotes in presence of trifluoperazine: washed promastigote cells (1×10^7/ml) in PBS were preincubated for 20 min in test tube in presence of 10 mM glucose. After 20 min cell suspensions were placed in the electrode chamber and the oxygen uptake recorded in a Gilson oxygraph using an oxygen-specific (Clarke type) electrode. Arrow indicates the time of addition of drugs (a) 5 µl DMSO, (b) 20 µM Trifluoperazine, and (c) 32 µM Trifluoperazine

growth of the organism. The treated cells were morphologically unaffected. Further, nonspecific leakage of cytoplasmic marker enzymes such as G-6-PDH could not be detected. Fura 2-AM, another membrane permeable Ca^{2+}-chelator, had earlier been shown by fluorometric techniques to effectively chelate internal cytoplasmic Ca^{2+} ion (Philosoph & Zilberstein 1989). Results with the ionophore and with Quin 2-AM suggest that disturbance of internal cytoplasmic free Ca^{2+} concentration has significant effect on cell division of the promastigote form of the organism.

Effect of Modulators of Ca^{2+} Metabolism on growth and Metabolism of ***L. donovani*** *Promastigote*

A large number of pharmacologically well-defined compounds are now available that have significant effects on Ca^{2+} metabolism. Table 1 summarizes the effect on the growth of *L. donovani* promastigotes of a few such model compounds. Phenothiazine group of drugs like chloropromazine or trifluoperazine are known to act as calmodulin antagonists in several eukaryotic systems (Gietzen et al. 1981). These drugs were found to arrest cell division and growth at moderately high concentrations. Calmidazolium, a anti-mycotic miconazole derivative and a more specific antagonist for calmodulin (Gietzen et al. 1982) had similar effect. W-7, the prototype of a series of napthalene sulfonamide compounds that are also known to have their pharmacological effect as calmodulin antagonists were also shown to have moderate leishmanicidal effect. at least at the initial stage of growth. In contrast, nifedipine, a channel-blocker for Ca^{2+} did not have any effect on the growth of the organism.

Further study indicated that both the phenothiazine group of drugs and calmidazolium had a somewhat generalized effect on the metabolism of the organism. Figure 2 shows that the oxygen consumption of this strongly aerobic organism is drastically and rapidly inhibited in presence of trifluoperazine. Similar results were obtained both with chloropromazine and calmidazolium (data not shown). In all these cases, a parallel rapid loss of motility was observed (data not shown) Interestingly, unlike the above antagonists of calmodulin, W-7 failed to show any inhibitory effect either on motility or on oxygen consumption.

Effect of some Ca^{2+} Modulators on Morphogenetic Transformation from Amastigote to Promastigote Form:

Purified *L. donovani* amastigotes from infected hamsters or BALB/c mice can be transformed to viable, flagellated promastigotes in a suitable medium. The methodology has been described in the text. The effect of some of the chelators and drugs were tested on this transformating system. The results are presented in figure 3. It is quite evident that unlike in case of promastigote division (table 1) nifedipine has a drastic inhibitory effect on transformation even at very low concentrations. Quin 2-AM also affected the transformation of amastigotes to promastigotes at significantly lower concentrations than that needed to affect promastigote division Trifluoperazine and W-7 have also strong inhibitory effects on this morphogenetic transformation. Similar results were obtained with chloropromazine and calmodizolium (data not shown). In general, the concentration of calmodulin antagonists needed to block the transformation of amastigote to promastigote form of the organism were almost the same as that required to arrest the growth of the latter form.

Discussion

The preliminary results obtained with some of the chelators, channel-blockers and calmodulin antagonists fall into a consistant pattern. Only compounds that disturb internal Ca^{2+} metabolism, such as A23187, Quin 2-AM (figure 1) trifluoperazine and calmidazolium and W-7 (table 1) have definite effect on *L. donovani* promastigote cell division. Ca^{2+} entry across the plasma membrane probably has no role to play in this process as EGTA and nifedipine failed to arrest cell division. The morphogenetic transformation, on the other hand, is almost certainly dependent on entry of Ca^{2+} from outside as it is totally blocked in presence of

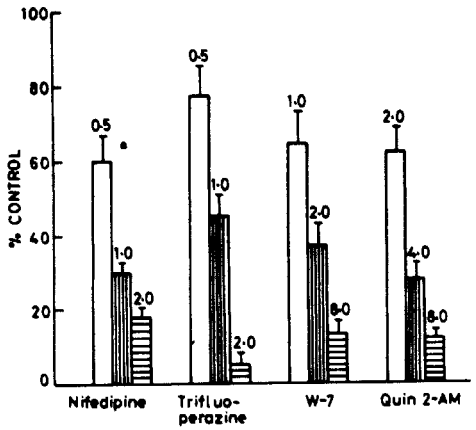

Figure 3 Effect of calcium chelator, channel blocker and calmodulin antagonists on the *in vitro* transformation of *L. donovani* amastigotes: The effect of these compounds were tested after 45 hr of incubation at 22°C by counting motile, flagellated promastigotes under microscope. The count in vehicle control represents 100% transformation. The specific compounds tested is indicated below the bars; values above the bars reprsent the concentration (μM) of that compound. The result shown are mean values of three sets of experiments

nifedipine (figure 3) and verapamil (data not shown). The inhibition of respiration (figure 2) and of flagellar motility by the phenothiazine group of drugs and by calmidazolium (data not shown) are suggestive of other site(s) of action for these agents apart from parasite calmodulin. This aspect of the problem is under further investigation. It is, however, sufficiently clear that homeostasis of Ca^{2+} and its entry across the plasma membrane are critical elements in transformation of the organism from the amastigote to promastigote form. Technical difficulties have so far prevented us from monitoring the effect of Ca^{2+} modulators on the pathogenic transformation of *L. donovani* promastigotes to amastigotes as well as amastigote cell division. Amastigote *in vitro* culture is not yet possible. The pathogenic transformation can only be carried out *in vitro* in a macrophage culture system where it is difficult to distinguish between the effect of the drug on the macrophage from the transformation process of the parasite. In spite of this incomplete picture for the whole

Table 1 *Effect of calcium channel blockers and anticalmodulin drugs on the growth of promastigote cells*

Item No	Drugs used	Number of cells × 10^5 per ml					
		0	17	23	46	73	92
1	Control (vehicle)	10 ± 1	11 ± 1	20 ± 1	80 ± 2	170 ± 3	140 ± 5
2	Nifedipine (5μM)	10 ± 1	12 ± 1	19 ± 1	78 ± 2	160 ± 3	180 ± 5
3	Nifedipine (10μM)	10 ± 1	12 ± 1	19 ± 1	78 ± 2	160 ± 3	180 ± 5
4	W-7 (5μM)	10 ± 1	12 ± 1	13 ± 1	19 ± 3	105 ± 7	145 ± 10
5	W-7 (10μM)	10 ± 1	10 ± 1	10 ± 1	17 ± 3	70 ± 5	125 ± 10
6	Trifluoperazine (0.5μM)	11 ± 1	11 ± 1	13 ± 1	51 ± 2	75 ± 7	110 ± 10
7	Trifluoperazine (1.0μM)	10 ± 1	10 ± 1	10 ± 1	7 ± 5	7 ± 5	5 ± 10
8	Calmidazolium (0.5μM)	10 ± 1	10 ± 1	10 ± 1	11 ± 3	39 ± 3	80 ± 12
9	Calmidazolium (1.0μM)	10 ± 1	7 ± 2	5 ± 2	—	—	—

Figures are the mean values of three sets of experiments ± S D

life cycle of the parasite, the data presented here shows that both Ca^{2+} entry and modulation of internal metabolism by free Ca^{2+} are essential features at least in the morphogenetic transformation of the amastigote to promastigote form.

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