Intracellular Ca\(^{2+}\) Pool Content and Signaling and Expression of a Calcium Pump Are Linked to Virulence in *Leishmania mexicana amazonensis* Amastigotes*

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during invasion, and the expression of a calcium pump that was cloned and sequenced.

EXPERIMENTAL PROCEDURES

Culture Methods—Two cloned populations of virulent (clone 12-D3) and avirulent (clone 250-HA) parasites were obtained as described (18) from the same isolate of *L. mexicana amazonensis* (RAT/BA/72/LV78) that was originally isolated in 1972 by Lainson and Shaw (see Ref. 19), passed in Syrian golden hamsters (R. S. Bray, Imperial College Field Station, Ascot, Berks, UK), and subsequently maintained in the laboratory of one of the authors (K.-P.C.) since 1979. One population of promastigotes was maintained in culture for more than 5 years and was subpassaged 250 times before cloning (250-HA), and the other was kept in liquid *N*. since 1980 and was cultured for 12 passages before cloning (12-D3). Cloning was done by the limiting dilution method of Dwyer (20). No significant differences were observed in the growth of these promastigote populations (data not shown). Amastigotes were obtained after passage of each cloned population of promastigotes into Grace's medium (21) with 20% heat-inactivated fetal calf serum, pH 5.25, at 33 °C. This medium was also used to maintain the amastigotes. Promastigotes from *L. mexicana amazonensis* as well as those from *L. donovani* (S2 strain), *Leishmania tropica* (LRC-L39 strain, courtesy of Dr. G. Trager, University of Granada, Spain), and *Leishmania major* (Friedlin strain, courtesy of I. Miller, University of Notre Dame) were grown in SDM-79 medium (22), pH 7.2, containing 10% fetal calf serum, at 26 °C. *Crichtidias desouzai* (CT-IOC 109, courtesy of Dr. M. de Sousa, Brazil) were grown at 26 °C in a liquid medium containing brain-heart infusion (37 g/liter) and hemin chlorohydrate (20 mg/liter dissolved in 50% triethanolamine). Two days after inoculation, cells were collected by centrifugation. All the cells were washed twice with medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml chloramphenicol, and 10% heat-inactivated fetal calf serum at 35 °C in a 5% CO2 incubator. Infectivity was calculated as the mean lesion diameter. Each experiment was repeated at least three times with different cell preparations, e.g. Figs. 3–6.

Cell Permeabilization—Variations in free Ca2+ concentrations in permeabilized cells were monitored by measuring the changes in the absorbance spectrum of arsenazo III (9, 10), using the SLM Aminco DW2000 spectrophotometer at the wavelength pair 675–685 nm.

Nucleic Acid Analysis—DNA was isolated by standard procedures (26). Total RNA was isolated by Trizol reagent following the manufacturer's recommendations (Life Technologies, Inc.). The polyadenylated RNA was obtained using a Poly(A)Tract mRNA isolation system. DNA was run in 0.8% agarose gels with TBE (9.5 mM Tris/boric acid, 2.0 mM EDTA, pH 8.0) and hemin chlorohydrate (20 mg/liter dissolved in 50% triethanolamine). Two days after inoculation, cells were collected by centrifugation. All the cells were washed twice with buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 5.5 mM glucose, and 50 mM Hepes buffer, pH 7.2). The final concentration of cells was determined using a Neubauer chamber. The protein concentration was determined by the biuret assay (23) in the presence of 0.2% deoxycholate.

*J774.A1* macrophages were cultured in Dulbecco's modified Eagle's medium with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum at 35 °C in a 5% CO2 incubator. Macrophages were infected with *L. mexicana amazonensis* at an estimated multiplicity of 10 parasites per macrophage in complete medium (24).

The infectivity of axenic amastigotes was determined essentially as described (24). Axenic amastigotes were taken from 3- to 4-day cultures. Amastigotes were recovered from the cultures by washing once with Grace's medium, then resuspended in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 5.5 mM glucose, and 50 mM Hepes buffer, pH 7.2) at a concentration of 107 cells/ml and incubated with 6 μM fura-2 AM for 30 min at 37 °C. After loading, the cells were washed three times with Dulbecco's phosphate-buffered saline. Culture medium containing parasites (loaded or not with fura-2) were added to loaded or unloaded macrophages, and the cells were observed by bright field or fluorescence microscopy. The images were collected with a system consisting of a CCD camera (model CH250; Photometrics Ltd., Tucson, AZ), an electronic unit (model CE 200A equipped with a 50-Hz 16-bit A/D converter), and a controller board (model NT 200; both from Photometric Ltd.). Images were acquired and evaluated by a software package (IPLab, Signal Analytics, Vienna, VA) on a Macintosh Quadra 840 AV computer (Apple Computer, Inc., Cupertino, CA). Cells were excited first at 340 nm and then at 380 nm. Point density readings were taken for each image, and a visual display of the 340/380 nm ratio was provided.

RESULTS

In Vivo and in Vivo Infectivity of Axenic Amastigotes—Fig. 1 shows typical growth curves of the axenic amastigotes about 10 subpassages after transformation. Clone 12-D3 and 250-HA had similar logarithmic 2-week plating, but clone 250-HA (12-D3) slowly after 3 days reaching a peak cell density lower than clone 250-D3. Doubling times varied between 18 and 24 h. The final cell densities of 12-D3 and 250-HA amastigotes were about 106/ml and 5 × 107/ml, respectively.

Infection of *J774.A1* macrophages by the two different clones of *L. mexicana amazonensis* was followed by determining the...
number of intracellular amastigotes. Fig. 2A shows the increase of 12-D3 amastigotes in macrophages. In contrast, 250-HA amastigotes disappeared with time. Fig. 2B shows that, when inoculated into hamsters, the 12-D3 amastigotes produced lesions that increased in size with time. The same number of 250-HA amastigotes produced no lesions.

\[ \text{Ca}^{2+} \text{, Increase Is Essential for Invasion of Macrophages by Virulent Amastigotes—Since a role for Ca}^{2+} \text{ in cell invasion by intracellular parasites has been postulated (2-4), we investigated the changes in [Ca}^{2+}]_i \text{ in amastigotes and promastigotes during their contact with J774.A1 macrophages at the single cell level. Parasite suspensions were added to glass slides with coverslips on which confluent monolayers of J774.A1 macrophages were grown. The host-parasite interaction was observed by phase contrast and fluorescence microscopy after incubation. When attached to macrophages, fura 2-loaded 12-D3 amastigotes were seen to increase their fluorescence in their cytoplasm as detected by ratio imaging (Fig. 3B). This increase in fluorescence was transient (<1 min) and asynchronous; not all amastigotes in contact with macrophages were highly fluorescent at the same time (Fig. 3B), although about 80% of the amastigotes observed in contact with macrophages were seen to increase their fluorescence at any time during the observation period. Interestingly, in most cases, the amastigotes with elevated Ca\textsuperscript{2+} were surrounded by macrophage pseudopodia (Fig. 3A). No changes could be detected in either avirulent (250-HA) amastigotes or promastigotes treated under the same conditions.

When 12-D3 amastigotes preloaded with the intracellular Ca\textsuperscript{2+} chelator quin 2/AM (50 \mu M) were used to infect J774.A1 macrophages for 1 h, their invasion rate was lower than controls (Table I). To investigate if this impairment was due to a lowering of cytosolic Ca\textsuperscript{2+} level, we loaded the amastigotes with another intracellular Ca\textsuperscript{2+} chelator, BAPTA/AM, which has a similar molecular structure and a comparable affinity for Ca\textsuperscript{2+} as does quin 2/AM, but has weak fluorescence emission (30). We could then use fura 2/AM as an indicator for cytosolic Ca\textsuperscript{2+} in cells preloaded with BAPTA/AM and exposed to NH\textsubscript{4}Cl and ionomycin, compounds capable of increasing [Ca\textsuperscript{2+}]_i in control preparations (see below). Under these conditions, Ca\textsuperscript{2+} concentration in control cells was 34 \pm 9 nM (n = 3). After exposure to NH\textsubscript{4}Cl/ionomycin for 5 min (Fig. 4B, control), the cytosolic Ca\textsuperscript{2+} concentration was 300 \pm 68 nM (n = 9). No significant changes were observed in the [Ca\textsuperscript{2+}]_i of BAPTA/AM-loaded amastigotes after exposure to NH\textsubscript{4}Cl/ionomycin under similar conditions (Fig. 4B, BAPTA). Similar to pretreatment with quin 2/AM, pretreatment with BAPTA/AM partially impaired the ability of amastigotes to invade macrophages (Table I). As a control for the possible toxicity of the intracellular chelators, amastigotes were loaded with half-BAPTA/AM, a compound of similar structure to BAPTA/AM but with no Ca\textsuperscript{2+} chelating action (30). No chelation of intracellular Ca\textsuperscript{2+} (Fig. 4B, half-BAPTA, dashed line) or decrease in the invasion rate of amastigotes was detected using this compound (Table I).

In conclusion, an increase in the [Ca\textsuperscript{2+}]_i occurs in virulent amastigotes when they attach to macrophages, and this is required for invasion.

\[ \text{Ca}^{2+} \text{, Increase in Macrophages Upon Contact with Virulent Promastigotes Is Not Essential for Invasion—To investigate if changes in [Ca}^{2+}]_i \text{ also occurred in the host cells upon contact with Leishmania, as it has been reported to occur in the case of infections with other intracellular parasites (see Ref. 3 for review), macrophages were loaded with fura 2 as described under “Experimental Procedures,” and changes in [Ca}^{2+}]_i \text{ were followed by digital imaging fluorescence microscopy. About 3 min after addition of virulent promastigotes (12-D3) (Fig. 5A), but not of any other stage of Leishmania (Fig. 5B and not shown), a transient increase in [Ca}^{2+}]_i \text{ was detected in the macrophages which then returned to basal levels. However, loading of the macrophages with BAPTA-AM, quin-2-AM, or half-BAPTA-AM as described before (Table I) did not change the rate of infection with either virulent or avirulent promastigotes or amastigotes (data not shown). In conclusion, a transient increase in [Ca}^{2+}]_i \text{ occurs in macrophages after their exposure to virulent promastigotes, and this is not essential for invasion, and no changes are detected in the presence of amastigotes.

Intracellular Ca\textsuperscript{2+} Pool Content in L. mexicana amazonensis—Since the Ca\textsuperscript{2+} content of intracellular stores exerts a profound control over cell growth, the progression of cells...
Ca2⁺, Content, Signaling, and Expression of a Calcium Pump

such protonophoric weak base effects on acidic compartments are well established for endocytic compartments (33). Fig. 6B shows that the addition of NH4Cl to L. mexicana amazonensis promastigotes caused an increase in [Ca2⁺]i, which was dose-dependent (not shown). The effect was not due to changes in osmotic pressure since no change in [Ca2⁺]i was observed upon the addition of up to 40 mM NaCl in the absence of an isotonic correction, and ammonium was concluded to be the active ion since (NH4)2SO4 produced identical results (data not shown).

Since these results were obtained in the nominal absence of extracellular calcium (1 mM EGTA was added), they indicate Ca2⁺ release from an intracellular compartment. The effects of ionomycin on the NH4Cl-induced rise of [Ca2⁺]i was investigated to identify the cellular origin of the Ca2⁺ mobilized by NH4Cl (13, 15, 34, 35). The addition of ionomycin (0.26 μM) to promastigotes previously exposed to NH4Cl (20 mM) caused a second rise in [Ca2⁺]i (Fig. 6B). Similar results were obtained when the order of additions was reversed (Fig. 6A). This indicates the existence in these cells of an ionomycin-sensitive pool that needs pH gradient neutralization in order for ionomycin-induced Ca2⁺ transport to be effective (35). This is because ionomycin binds essentially no calcium below pH 7.0 (36). So, it will not carry calcium out of acidic compartments because protons would compete with calcium for binding of the ionophore at the inner face of the membrane (34).

To further demonstrate the presence of acidocalcisomes in L. mexicana amazonensis, we incubated fura 2-loaded cells in the presence of nigericin (a H+/K⁺ exchanger (37) (Fig. 6D) or bafilomycin A1, a specific inhibitor of the vacuolar H⁺-ATPase (38) (Fig. 6F)) in the absence of extracellular Ca2⁺. In all cases there was an increase in [Ca2⁺]i, which was further elevated by the addition of ionomycin. Similar results were observed when the order of additions was reversed (Fig. 6, C and E), although bafilomycin A1 addition after ionomycin caused a slower Ca2⁺ release (Fig. 6E). In agreement with these results bafilomycin A1 addition before (Fig. 6F, BAF) or after NH4Cl addition (data not shown) also caused a slower Ca2⁺ release.

These results indicate that L. mexicana amazonensis cells have an acidic compartment that possesses a significant amount of Ca2⁺ and is sensitive to H⁺/K⁺ exchangers, inhibitors of the H⁺-ATPase, and weak bases, i.e. acidocalcisomes (12–16). Further characterization of these organelles is under-
The steady-state basal [Ca\(^{2+}\)] of the *Leishmania* parasites in the presence of 1 mM EGTA was not significantly different between different stages with the exception of a slightly higher concentration in HA amastigotes (amastigotes 12-D3, 34 ± 9 nM (n = 10); amastigotes 250-HA, 53 ± 5 nM (n = 10); promastigotes 12-D3, 36 ± 4 nM (n = 10), promastigotes 250-HA, 32 ± 7 nM (n = 10)). However, the amount of releasable Ca\(^{2+}\) was at least 3-fold higher in virulent amastigotes (12-D3) as compared with all the other forms (Fig. 4A shows only a comparison between amastigotes and promastigotes of the 12-D3 clone, for clarity), and after addition of ionomycin/NH\(_4\)Cl the [Ca\(^{2+}\)] of virulent amastigotes reached values of about 300 ± 68 nM (n = 9) or about 10-fold greater than basal steady-state values, whereas the values obtained with the rest were much lower although slightly higher in avirulent amastigotes than in promastigotes (amastigotes 250-HA, 102 ± 22 nM (n = 10); promastigotes 12-D3, 69 ± 15 nM (n = 8); promastigotes 250-HA, 41 ± 4 nM (n = 8)).

To learn whether there was a correlation between the Ca\(^{2+}\) content of the virulent amastigotes and their infectivity, we depleted their intracellular Ca\(^{2+}\) stores as indicated before (Fig. 4) and measured their infectivity to J774.A1 macrophages; the cells were treated with 1 mM ionomycin and 20 mM NH\(_4\)Cl for 10 min at room temperature in buffer A containing 1 mM EGTA, washed twice, and incubated in the same buffer for 30 min in the presence of J774.A1 macrophages as described in Table I. The number of amastigotes per 100 macrophages decreased by 45% (50 ± 13.4 amastigotes/100 macrophages) as compared with untreated controls (91 ± 29 amastigotes/100 macrophages; mean ± S.D. from three different experiments). Although treated cells were viable at the end of the 10-min treatment, as judged by the trypan blue staining, when the parasites were left in the Ca\(^{2+}\)-depleting buffer for 20 min or...
Figure 6. Effect on intracellular calcium concentration of compounds that produce alkalinization of acidic compartments and ionomycin. L. mexicana amazonensis promastigotes (12-D3, 2×10^7 cell/ml) were incubated in the presence of buffer A containing 1 mM EGTA. Additions were as follows: 0.26 μM ionomycin (ION), 1 μM bafilomycin A1 (BAF), 1 μM nigericin (NIG), or 20 mM NH₄Cl. Other experimental conditions are indicated under “Experimental Procedures.”

more they started to lyse thus indicating a toxic effect of prolonged treatment of the parasites with that buffer.

Cloning of an Organelle-type Ca²⁺-ATPase Gene—The higher amount of releasable Ca²⁺ in the virulent amastigotes seen (Figs. 3–4) could be due to a more active Ca²⁺ uptake, a reduced Ca²⁺ leak from intracellular stores under nonstimulating conditions, or a greater storage volume. An enhanced uptake of Ca²⁺ seems likely since this could be mediated by the expression of an endoplasmic reticulum calcium pump that has been linked to the control of cell growth (39). We therefore investigated the expression of organelle-type Ca²⁺-ATPases in these parasites.

Ca²⁺-ATPases are members of a family of P-type ion pumps that contain conserved domains. Degenerated oligonucleotides corresponding to two of these domains, a phosphorylation site and a site involved in ATP binding (27, 28), were used to PCR-amplify specific sequences from L. mexicana amazonensis genomic DNA. The PCR products were cloned and sequenced. Analysis of the deduced partial amino acid sequence of these clones revealed that a 1.2-kb PCR clone had the best score of sequence identity (55–65%) with the organelle-type Ca²⁺-ATPases (or sarcoplasmic-endoplasmic reticulum (SERCA) Ca²⁺-ATPases), such as those from T. brucei (40), Plasmodium falciparum (41), and rabbit (42). This clone also hybridized to restriction enzyme-digested T. cruzi and T. brucei genomic DNA on Southern blots under low stringency conditions. Therefore, this clone was most likely encoding an organellar type Ca²⁺-ATPase.

To obtain the complete gene, this PCR clone was used as a probe to screen a lambda EMBL3 genomic library of L. mexicana amazonensis. Southern hybridization of EcoRI-digested genomic DNA with the 1.2-kb clone revealed a single ~20-kb hybridization band. Seventeen positive clones were obtained. Sequencing of these clones revealed a complete open reading frame (lmaa1, Fig. 7) with 3093 nucleotides. The DNA sequence of the 1.2-kb PCR product was identical to the corresponding region of the gene obtained from the lambda EMBL3 genomic library except for one nucleotide. This was apparently due to the use of degenerate primers for the PCR. According to the initiation codon ATG predicted (Fig. 7), the open reading frame codes for a protein of 1031 amino acids with a calculated molecular mass of 113.03 kDa.

Structure of the Coding Region and Genomic Organization of lmaa1—Analysis of the Lmaa1 amino acid sequence (Fig. 7) showed that this gene product contains all the conserved subdomains and invariant residues found in other P-type ATPases, such as the phosphorylation and ATP-binding domains (27, 28). Hydropathy analysis of the deduced amino acid sequence (not shown) revealed a profile very similar to those of SERCA pumps containing 10 transmembrane domains. A TFASTA search of protein data bases showed that Lmaa1 was most closely related to the SERCA-type Ca²⁺-ATPases, with 64.75% identity (76.46% similarity) over 1015 amino acids to the one of cardiac sarcoplasmic reticulum (SERCA) Ca²⁺-ATPase of cardiac sarcoplasmic reticulum with phosphomodulin binding found near the C terminus of all mammalian plasma membrane Ca²⁺-ATPases (PMCA) isoforms (44), consistent with the Ca²⁺-ATPases from other lower eukaryotic organisms, i.e. TBA1 of T. brucei (45), tca1 of T. cruzi (46), and pat1 of Dictyostelium discoideum (47) and three gene products of Plasmodium falciparum (41, 48, 49).

The amino acid sequence Lys-Asp-Asp-Lys-Pro-Val^402, which corresponds to the conserved amino acid sequence associated with calmodulin binding found near the C terminus of all mammalian plasma membrane Ca²⁺-ATPase (PMCA) isoforms (44), is absent in Lmaa1. Interestingly, the residues located in transmembrane segment 3 important for thapsigargin binding to SERCA Ca²⁺-ATPases (51) are different in Lmaa1 (11 out of 20 residues in segment 3 are different as compared with SERCA pumps). In agreement with these re-

sults, we were unable to detect any significant increase in [Ca$^{2+}$]i, in turr 2-loaded D3 amastigotes in the presence of low concentrations of thapsigargin (0.1–1 µM), a known inhibitor of SERCA pumps (52).

Genomic DNA was digested with several restriction enzymes selected to demonstrate genome copy number and hybridized at high stringency with a 1375-bp PstI fragment of lmaa1 (see Fig. 8C). Each of the seven different restriction enzymes used produced a single band that varied in size (Fig. 8A). This suggests that lmaa1 is present as a single copy gene in L. mexicana amazonensis. To further confirm this, additional enzymes were selected to cut once both outside (double digestion with BamHI and EcoRI and single digestion with XbaI; lanes 8 and 9, respectively) and inside (PstI, lane 10) the predicted coding region of lmaa1. The digests were probed with a 4.3-kb XbaI fragment containing the complete coding region plus 0.7 kb of the upstream flanking region and 0.6 kb of the downstream flanking region (Fig. 7C). A 6.6-kb single band was detected in the sample digested with BamHI and EcoRI, and a single 4.3-kb band with XbaI (lane 9). These fragments are consistent in size with those expected from the DNA sequence obtained.

PstI-digested samples, showing a single 1.4-kb band, were analyzed with a PstI fragment of the lmaa1 coding region (Fig. 7B). A single 1.6-kb band was detected in all samples digested with PstI (lanes 1–3), indicating that the cloned DNA fragment is a single copy molecule. These results are consistent with those obtained from Southern blot analysis (Fig. 8A), confirming the single-copy nature of lmaa1.

**Fig. 7. Nucleotide and predicted protein sequence of Lmaa1.** Amino acid residues are numbered in the left margin. Nucleotides are numbered in the right. The amino acid sequences corresponding to the highly conserved catalytic autophosphorylation and ATP-binding domains employed in the design of degenerated oligodeoxyribonucleotides for PCR are in bold. Ten transmembrane spanning domains identified by hydrophathy analysis are underlined. High affinity Ca$^{2+}$-binding sites identified in SERCA pumps (43) are in bold italic. The TTG stop codon for lmaa1 is marked by an asterisk.
The use of digitonin to permeabilize the plasma membrane of different trypanosomatids has allowed the detection of bands of soluble protein (mean ± S.D. from two different experiments).

**FIGURE 8.** Southern blot analysis of the lmaa1 gene in genomic DNA of *L. mexicana amazonensis*, *L. donovani*, *L. tropica*, and *L. major*. Total genomic DNA (10 μg/lane) was digested with various restriction enzymes and electrophoresed through agarose gels and transferred to a Zeta-Probenylon membrane as described under “Experimental Procedures.” The blots were hybridized with a 1375-bp fragment of *lmaa1* gene and washed at high stringency. Size markers correspond to the fragments resulting from λ-DNA digested with *Hin*-III, *L. mexicana amazonensis* genomic DNA digested with the following restriction enzymes: lane 1, BamHI; lane 2, EcoRI; lane 3, HindIII; lane 4, PstI; lane 5, SacI; lane 6, PvuII; lane 7, KpnI; lane 8, *L. mexicana amazonensis* genomic DNA digested with the following restriction enzymes: lane 8, BamHI/EcoRI double digest; lane 9, XbaI; lane 10, PstI; and digestion with PstI of genomic DNA from *L. donovani* (lane 11), *L. tropica* (lane 12), and *L. major* (lane 13). Band when probed with the 1375-bp PstI fragment (lane 4), allowed the detection of bands of ~1.4, ~3, and ~3.5 kb when probed by the 4.3-kb XbaI fragment. These latter two bands represent both flanking regions of the 1375-bp PstI fragment. All these results are consistent with the conclusion that *lmaa1* is present as a single copy gene.

The *lmaa1* gene is also present in other *Leishmania* spp. (Fig. 8B, lanes 11–13). Southern blot analysis of PstI-digested genomic DNA from *L. donovani* (lane 11), *L. tropica* (lane 12), and *L. major* (lane 13) was carried out at high stringency using genomic DNA digested with the following restriction enzymes: lane 8, BamHI/EcoRI double digest; lane 9, XbaI; lane 10, PstI; and digestion with PstI of genomic DNA from *L. donovani* (lane 11), *L. tropica* (lane 12), and *L. major* (lane 13).

**Overexpression of lmaa1 in Virulent Amastigotes—Northern blot analysis showed a single ~3.5-kb transcript in the two life cycle stages of both virulent and avirulent *L. mexicana amazonensis* (Fig. 9A).** The expression of this transcript is up-regulated by 2–4-fold in the 12-D3 amastigotes based on the intensity of the 3.5-kb bands. All samples were equally loaded, as seen by hybridization with a full-length β-tubulin gene (53) (Fig. 9B). An increased expression of this gene could, at least in part, explain the elevated amount of releasable Ca<sup>2+</sup> seen with virulent amastigotes (Fig. 4A) and the Ca<sup>2+</sup> signaling during their invasion of macrophages (Fig. 3).

**Permeabilization Experiments with L. mexicana amazonensis Amastigotes—**The use of digitonin to permeabilize the plasma membrane of promastigotes without acidocalcisomes has allowed the identification of the mechanisms involved in Ca<sup>2+</sup> transport in acidocalcisomes (12–15). The results were consistent with the presence of a Ca<sup>2+</sup>/H<sup>+</sup>-ATPase system pumping Ca<sup>2+</sup> into these acidic vacuoles (12, 15). To find out if that was also the case with *L. mexicana amazonensis* amastigotes, we treated these cells with digitonin in the presence of arsenazo III to follow changes in ambient Ca<sup>2+</sup>. Addition of 20 μM digitonin to either virulent or avirulent amastigotes caused an immediate Ca<sup>2+</sup> release (Fig. 10). No Ca<sup>2+</sup> uptake could be detected even reducing the digitonin concentration to 5 μM (not shown) or using 10 μg of filipin/ml as an alternative permeabilizing agent (Fig. 10, V, Fil.) and performing the incubations in the presence of a mitochondrial substrate (2 mM succinate) or ATP (1 mM, data not shown) (9, 10, 12–15). Although filipin addition produced a slower Ca<sup>2+</sup> release than digitonin, the final set point was similar (Fig. 10). It is interesting to note that Ca<sup>2+</sup> release was biphasic (Fig. 10) possibly indicating release from different intracellular pools of different susceptibilities to the detergents. In this regard, a similar digitonin-induced Ca<sup>2+</sup> release has been reported in promastigotes of *Leishmania braziliensis* (6). This effect could be due to a similar sterol composition of the plasma membrane and the intracellular membranes of amastigotes of *L. mexicana amazonensis*, in contrast to what occurs with either *T. brucei* (12) or *T. cruzi* (15) or promastigotes of different *Leishmania* spp. (9, 10) where selective permeabilization of the plasma membrane is possible due to its different sterol composition as compared with that of intracellular compartments. This effect precluded any investigation of the possible mechanisms of Ca<sup>2+</sup> uptake in acidocalcisomes of these parasites. However, it was possible to confirm that the total releasable Ca<sup>2+</sup> of *L. mexicana amazonensis* amastigotes (21.5 ± 2.3 nmol/mg protein) was 3.8-fold higher than that present in avirulent amastigotes (5.7 ± 0.9 nmol/mg protein) (mean ± S.D. from two different experiments).

**DISCUSSION**

We have shown that a significant amount of Ca<sup>2+</sup> within *L. mexicana amazonensis* promastigotes and amastigotes is located in an acidic compartment, i.e. acidocalcisome (12–16), that is sensitive to H<sup>+</sup>/K<sup>+</sup> exchangers, inhibitors of the H<sup>+</sup>-ATPase, and weak bases. These results extend previous findings of the presence of acidocalcisomes in other trypanosomatids (12–15). Several drugs effective against *Leishmania* (amino acid esters, 54) or other trypanosomatids (chloroquine (55) and daunomycin (56)) have been shown to accumulate in acidic compartments (54–56). The chemotherapeutic effects of these agents may involve an alteration of Ca<sup>2+</sup> homeostasis through disruption of this Ca<sup>2+</sup>-rich acidic compartment.

Our data also show that *L. mexicana amazonensis* amastigotes possess more releasable Ca<sup>2+</sup> than promastigotes. The concentration of Ca<sup>2+</sup> in amastigote-containing phagolysosomes in macrophages is not known, but if it were similar to that in the cytosol (about 0.1 μM) it would be dramatically different from the concentration of Ca<sup>2+</sup> to which extracellular parasites are exposed (about 1 mM). The higher amount of releasable Ca<sup>2+</sup> in amastigotes would then indicate an adaptation to an intracellular environment. Alternatively, it has been indicated that the parasitophorous vacuoles of *Leishmania* contain a constituting part of the phagosome-lysosome vacuolar apparatus open to the extracellular membrane from which exogenous substances can be brought into contact with amastigotes via pinocytosis and/or phagocytosis of macrophages during the entire period of infection (57). If this were the case, no difference in Ca<sup>2+</sup> would be expected to exist between the extracellular space and the phagolysosomes. The higher amount of releasable Ca<sup>2+</sup> in amastigotes would then indicate a specific function for Ca<sup>2+</sup> in these forms, such as in signaling during invasion.

Axenic in vitro cultivation of amastigotes has been previously reported for *Leishmania mexicana* (58), *Leishmania pi-fanoi* (59), *Leishmania panamanensis* (60), *L. braziliensis* (60), *L. major* (61), *L. donovani* (61, 62), and *L. mexicana amazonensis* (63). In this study, two clones of *L. mexicana amazonensis* were adapted to grow axenically as amastigotes. A clear link between their intracellular Ca<sup>2+</sup> pool content, Ca<sup>2+</sup> signaling...
be important for thapsigargin binding and that are conserved in all SERCA Ca$^{2+}$-ATPases (51) are different in Lmaa1, and this correlates with the absence of effect of low concentrations of thapsigargin on the [Ca$^{2+}$], of fura 2-loaded cells. A comparison of the sequence of this transmembrane segment M3 in Lmaa1 with those of other Ca$^{2+}$-ATPases indicates that Lmaa1 shares 9 out of 20 amino acid residues with SERCA pumps and 14 out of 20 residues with T. brucei Ca$^{2+}$-ATPase TBA1 (45). Interestingly, TBA1 only shares 9 out of 20 amino acid residues with SERCA pumps, and 8 of these 9 amino acid residues are in common with Lmaa1 (see Scheme 1 showing the alignment of Lmaa1 with TBA1, SERCA1, SERCA2, and SERCA3; and indicate identical and similar amino acid residues, respectively).

![Figure 9: Expression of Lmaa1 mRNA. A, five µg of poly(A)$^+$ RNA isolated from promastigotes (lane 1) and amastigotes (lane 2) from virulent L. mexicana amazonensis (clone 12-D3) or from promastigotes (lane 3) and amastigotes (lane 4) from avirulent L. mexicana amazonensis (clone 250-HA) were size-fractionated on an agarose gel, transferred to a nylon membrane, and probed at high stringency with the 1.2-kb PCR fragment. Relatively equal amounts of RNA in each lane were viewed under UV light. Size markers correspond to a 0.24–9.5-kb RNA ladder (Life Technologies, Inc.). B, the membrane was stripped and reprobed with a full-length β-tubulin gene from L. mexicana amazonensis. C, the density ratio A/B.

![Figure 10: Effect of digitonin and filipin on endogenous Ca$^{2+}$ release from permeabilized amastigotes. 20 µM digitonin (Dig.), or 10 µg/ml filipin (Fil.) were added where indicated by the arrows to L. mexicana amazonensis 12-D3 (virulent) amastigotes (V, Dig. or V, Fil., 0.43 mg of protein/ml) or 250-HA (avirulent) amastigotes (A, Dig., 0.73 mg of protein/ml) in a buffer containing 130 mM KCl, 1 mM MgCl$_2$, 2 mM K$_2$HPO$_4$, 20 mM Tris-HCl buffer, pH 7.4, 40 µM arsenazo III at 33 °C. V, control were 12-D3 amastigotes without any detergent added. V, Fil. and A, Dig. tracings were superimposed for the first 7 min. Note the biphasic Ca$^{2+}$ release under all treatments. Results shown are from representative experiments.

Since the TBA1 product is reportedly sensitive to thapsigargin (40), it is interesting to compare the amino acid sequences in the corresponding region between TBA1 and Lmaa1. Two residues are completely different: a Gly$^{271}$ → Lmaa1 replaces a Lys$^{261}$ in TBA1, and a Phe$^{279}$ → Lmaa1 replaces a Val$^{269}$ in TBA1. On the other hand, substitutions of Lys$^{261}$ in TBA1 by histidine in SERCA3 and of Val$^{269}$ in TBA1 by alanine in SERCA1, 2, and 3, preserved the sensitivity of the proteins to thapsigargin. This could suggest that the basic side chain amino acid lysine (or histidine) and the aliphatic side chain of the amino acid valine (or alanine) may be important for thapsigargin binding and that the changes in these positions in Lmaa1 abolish the sensitivity to thapsigargin. Alternatively, the method that we used to detect thapsigargin sensitivity may not have the same sensitivity as when the enzyme is overexpressed such as in the case of the T. brucei enzyme (40).

Although the increase in Ca$^{2+}$ pump mRNA does not prove that increased pump protein is responsible for the increased Ca$^{2+}$ content in virulent amastigotes, it is highly suggestive of this possibility. The presence of a H$^+$-countertransporting...
Ca\(^{2+}\)-ATPase involved in Ca\(^{2+}\) sequestration that resides in the acidicocalcisomes of other trypanosomatids such as T. brucei (12) and T. cruzi (15) has been postulated. The vanadate sensitivity of all non-mitochondrial Ca\(^{2+}\) uptake by permeabilized trypanosomatids (12, 15) argued against the involvement of the type of Ca\(^{2+}\)/H\(^{+}\) antiporter believed to exist in the vacuoles of fungi and higher plants (64, 65). However, it has also been demonstrated (12) that, as occurs with L. mexicana amazonensis (Figs. 4 and 6), the inside pH gradient due to the vacuolar proton pump facilitates Ca\(^{2+}\) uptake and retention. Therefore, we cannot rule out that overexpression of a vacuolar proton pump could also occur in virulent amastigotes and be responsible in part for their higher Ca\(^{2+}\) content. Alternatively, a reduced H\(^{+}\) permeability of the acidicocalcisomes of the virulent parasites could also account for part of their increased Ca\(^{2+}\) content. In this regard, Ca\(^{2+}\) release from the acidicocalcisomes of T. brucei procyclic trypomastigotes via a Ca\(^{2+}\)/H\(^{+}\) antiporter has been shown to be stimulated by Na\(^{+}\) via a Na\(^{+}/\)H\(^{+}\) antiporter (14). Further work is necessary to clarify the role of all these pumps and exchangers in Ca\(^{2+}\) uptake and retention by trypanosomatid acidocalcisomes. However, the high sensitivity of L. mexicana amazonensis to digitonin or filipin permeabilization (Fig. 10) precludes the use of these parasites as a model for these studies.

It has been demonstrated that the Ca\(^{2+}\) content of intracellular stores exerts a profound control over cell growth and the progression of cells through the cell cycle (31, 32), and that growth changes can result from the inability of Ca\(^{2+}\) to be pumped into the intracellular stores (32). Depletion of Ca\(^{2+}\) from within the intracellular stores has considerable inhibitory effect on the folding and processing of proteins (66, 67). It is clear that a range of Ca\(^{2+}\)-binding proteins in these stores are involved in not just the storage of Ca\(^{2+}\) within the lumen but are also functioning as “Ca\(^{2+}\)-dependent chaperones” to assist the folding and correct assembly of proteins (39). An alternative or additional possibility is that the decrease in Ca\(^{2+}\) within intracellular stores in the avirulent amastigotes (250-MA) may preclude important Ca\(^{2+}\) signals. These signals may be generated through release of Ca\(^{2+}\) from the stores essential for the parasites to progress through the cell cycle and maintain growth or to replicate successfully within mammalian cells. Although 250-MA amastigotes have the capacity to invade macrophages in cultures or animals, they have lost their ability to replicate intracellularly and develop a successful infection. The results provide not only further support to the postulated link between intracellular Ca\(^{2+}\) pool content, expression of Ca\(^{2+}\)-ATPases, Ca\(^{2+}\) signaling, and cell growth in eukaryotic cells (31, 32, 39) but also evidence for an important link with virulence of intracellular parasites.

REFERENCES

Intracellular Ca$^{2+}$ Pool Content and Signaling and Expression of a Calcium Pump Are Linked to Virulence in *Leishmania mexicana amazonesis* Amastigotes

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