Biochemical and Biophysical Research Communications 430 (2013) 1091–1096

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of a sphingosine-sensitive Ca^{2+} channel in the plasma membrane of *Leishmania mexicana*

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ARTICLE INFO

Article history:
Received 4 December 2012
Available online 19 December 2012

Keywords:
Leishmania
Calcium channel
Trypanosomatids
Sphingosine
Ca^{2+}

ABSTRACT

The disruption of the intracellular Ca^{2+} homeostasis of *Leishmania mexicana* represents a major target for the action of drugs, such as amiodarone and miltefosine. However, little is known about the mechanism of Ca^{2+} entry to these cells. Here we show the presence of a Ca^{2+} channel in the plasma membrane of these parasites. This channel has many characteristics similar to the human L-type voltage-gated Ca^{2+} channel. Thus, Ca^{2+} entry is blocked by verapamil, nifedipine and diltiazem while Bay K 8644 opened this channel. However, different to its human counterpart, sphingosine was able to open this channel, while other well known sphingolipids had no effect. This fact could have important pharmacological implications.

1. Introduction

Leishmania mexicana, the causative agent of cutaneous and mucocutaneous leishmaniasis is a trypanosomatid. The parasite, after being inoculated in the bloodstream by a sandfly in the form of promastigotes, is taken actively by circulating macrophages, where the parasite transforms to the amastigote form. Then, after reaching a critical number, the host-cell is disrupted, discharging the amastigotes, which in turn invade other macrophages repeating the cycle and thus producing the illness [1].

It has been demonstrated that Ca^{2+} is involved in several functions in these parasites [2], including differentiation and host cell invasion [3,4]. The mechanisms involved in the intracellular Ca^{2+} regulation in these parasites are quite well described [1–3]. They possess a distinctive single mitochondria able to accumulate Ca^{2+} by an electrophoretic uniporter, using as driving force for Ca^{2+} uptake, the mitochondrial electrochemical potential [5]. These parasites also possess acidocalcisomes, interesting acidic organelles able to accumulate large amounts of Ca^{2+} [6]. Acidocalcisomes are very relevant concerning the bioenergetic of these parasites since they accumulate Ca^{2+} in combination with pyrophosphate, which is an energy source, alternative to ATP [2,3]. The endoplasmic reticulum is also involved in Ca^{2+} uptake [2,3]. At the plasma membrane, the parasite is able to extrude Ca^{2+}, due to the presence of a Ca^{2+}-ATPase [7]. However, little is known concerning the mechanisms of Ca^{2+} entry. It has been recently shown that many drugs of current use against this parasite exert their action through the disruption of its intracellular Ca^{2+} homeostasis. Thus, miltefosine, an alkyl-lysophospholipid of general use against leishmaniasis, is known to exert its leishmanicidal action through the opening of a not yet characterized plasma membrane Ca^{2+} channel [8]. Amiodarone and dronedarone, commonly used antiarrhythmic drugs, are known to strongly affect *L. mexicana* [8,9] and other trypanosomatids [10–12], such as *Trypanosoma cruzi*, the causative agent of Chagas disease. These drugs concern the Ca^{2+} homeostasis, affecting the acidocalcisomes, and also collapsing the electrochemical mitochondrial potential, thus inducing the release of Ca^{2+} to the cytoplasm [9,11,12].

Concerning the mechanisms of Ca^{2+} entry to these parasites, the presence of a Ca^{2+} channel in the plasma membrane should be warranted since these parasites are able to significantly change the intracellular Ca^{2+} content depending on different conditions, for example, during cell invasion [4]. However, information related to the presence of such a Ca^{2+} channel is scarce. It has been reported that arachidonic acid induces an intracellular Ca^{2+} increase in *L. mexicana* [13], but part was due to the release of the cation from intracellular organelles, such as mitochondria and acidocalcisomes, since it was also observed in the absence of extracellular Ca^{2+} [13]. Melittin, on the other hand, is able to induce a Ca^{2+} influx, which appears to depend on the activity of a PLD_2. Interestingly, based on *Leishmania major* genomic evidences, two genes putatively coding for a protein with similar characteristic to those of an L-type voltage-gated Ca^{2+} channel (VGCC) could be present in

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http://dx.doi.org/10.1016/j.bbrc.2012.12.033

these parasites [14]. In the present work we demonstrate the presence of a Ca^{2+} channel in the plasma membrane of *L. mexicana*, with many similarities with the human VGCC. Accordingly, nifedipìne and verapamil, classical inhibitors of this channel, known to affect the growth of *Leishmania sp*. [15], block the parasite Ca^{2+} channel. However, differently from its putative human counterpart, the *L. mexicana* Ca^{2+} channel is opened by the sphingolipid sphingosine (Sph), which is known to be present in these parasites [16,17].

2. Materials and methods

2.1. Chemicals

Sphingosine, ceramide, sphingosine-1-P and ceramide-1-P were purchased from Avanti Polar Lipids Inc. EGTA, digitonin, verapamil, nifedipine, diltiazem were from SIGMA. Fura 2-acetoxymethyl ester (FURA 2-AM) was purchased from Molecular Probes.

2.2. Culture of promastigotes of L. Mexicana

Promastigotes of *L. mexicana* were cultured in liver infusion – tryptose (LIT) medium supplemented with 10% inactivated fetal bovine serum under continuous agitation at 29 °C as previously reported [8].

2.3. Intracellular Ca^{2+} measurements

Promastigotes were loaded with the fluorescent ratiometric Ca^{2+} indicator Fura 2, to estimate variations on intracellular Ca^{2+} concentration, as described [9]. Briefly, 2×10^8 parasites were collected by centrifugation at 600*g* for 2 min and washed twice in PBS buffer plus 1% glucose. Then, the parasites were loaded with Fura 2-AM (6 μM), probenecid (12 μM) and pluronic acid (12 μM) in the same buffer at 29 °C, in the dark under continuous agitation for 4 h. The Fura 2-loaded parasites were washed twice by centrifugation and resuspended in Tyrode buffer. Resuspended parasites were placed in a cuvette under continuous stirring at 29 °C in a PerkinElmer 510 Spectrofluorimeter coupled to a fast-filter device that allows the alternating excitation at Ex 340 nm/380 nm [11]. Thus, the conditions of measurement were Ex 340 nm/380 nm and Em 510 nm, and the results were expressed as the ratio values of the λ Em at the two excitation wavelength.

3. Results

Different sphingolipids, such as ceramide, sphingosine (Sph), ceramide-1-P and sphingosine-1-P, are known to increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in many human cell lines [18,19]. In order to study the possible effect of these sphingolipids on *L. mexicana*, cultured promastigotes were loaded with the Ca^{2+} indicator Fura 2. In Fig. 1A it can be observed that addition of 20 μM Sph, concentration reported to exert its optimal effect on the $[Ca^{2+}]_i$ in human cell lines [18], is able to induce a large increase in the $[Ca^{2+}]_i$ of these parasites. Lower concentrations of Sph also increase the $[Ca^{2+}]_i$ of these parasites, but to a lesser extent in a dose-dependent manner (results not shown). For this reason we used 20 μM Sph along this work. Interestingly, other sphingolipids, such as ceramide, sphingosine-1-P and ceramide-1-P, at even higher concentrations than those known to increase the $[Ca^{2+}]_i$ in human cell lines [18,19], were not able to affect the intracellular Ca^{2+} content of these parasites (Fig. 1B). Digitonin was added in these experiments to allow the maximal Ca^{2+} entry to the cells. Since digitonin was able to further induce an increase in the Ca^{2+} fluorescence, the Ca^{2+} channel opened by Sph, similar to the human L-type VGCC, should be able to be inactivated.

Nifedipine, verapamil and diltiazem are the canonical inhibitors of the human L-type VGCC. To test these inhibitors on the effect observed by Sph on the $[Ca^{2+}]_i$, we added sequentially the different inhibitors prior to the addition of the sphingolipid. In Fig. 2A and B it can be observed that nifedipinde and verapamil, at concentration used to block the human VGCC, totally blocked the effect of Sph. Diltiazem (10 μM) also abolished the effect of Sph (result not shown).

A very specific agonist of the human L-type VGCC, BayK 8644, has been widely used for the characterization of its function [20]. In Fig. 2C it can be observed that this agonist was able to substitute Sph. Addition of the sphingolipid after BayK 8644 did not induce a further Ca^{2+} release. Accordingly, if Sph is added before the channel agonist, the latter is without effect (Fig. 2D).

We then studied whether the effect of Sph was due to a Ca^{2+} entrance from the extracellular milieu, or instead, it was consequence of its release from intracellular organelles. In Fig. 3A (Top), we showed that when EGTA was added to sequester Ca^{2+} from the extracellular solution, there was a drop in the fluorescence, probably because of the spontaneous release of some Fura 2 from the parasite. Under this condition, addition of Sph, instead of inducing a $[Ca^{2+}]_i$ increase, produced a dramatic fall. This result is compatible with the exit of the basal intracellular Ca^{2+} to the outer medium,

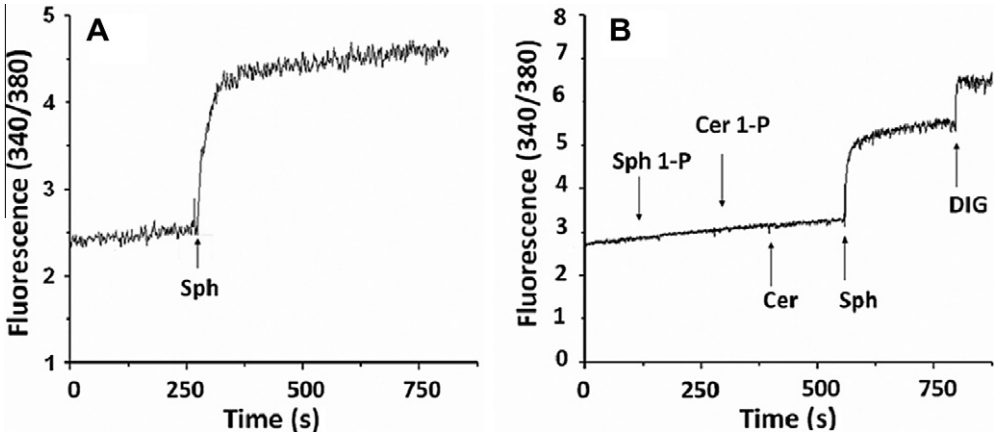

Fig. 1. Effect of different sphingolipids on the intracellular Ca^{2+} concentration of *L. mexicana*. Promastigotes of *L. mexicana* were loaded with Fura 2, as explained under "Experimentals". (A) Sphingosine (20 μM) was added (arrow) directly to the stirring cuvette in the presence of 2 mM $CaCl_2$. (B) Sphingosine 1-P (Sph 1-P, 20 μM), Ceramide 1-P (Cer 1-P, 20 μM), Ceramide (Cer, 20 μM), Sphingosine (Sph, 20 μM) and Digitonin (DIG, 30 μM) were added (arrows) directly to the stirring cuvette in the presence of 2 mM $CaCl_2$. Traces are representative of at least four independent experiments.

due to the presence of EGTA. This result also demonstrates that the channel opened by Sph is able to permit the Ca^{2+} fluxes in both directions, similar to its human analog. In order to study if verapamil could block this Ca^{2+} release, the channel blocker was added before Sph (Fig. 3B). Under this condition the effect of Sph was unaffected, demonstrating that verapamil can only obstruct the channel when acting from outside, probably acting resembling a stopper.

We then test if addition of Sph directly to the parasite population was able to show any discernible effect. We could observe that after 20 min of the addition of 5 μM of the sphingolipid to promastigotes of *L. mexicana*, the parasites started to take a rounded form (compare Fig. 4B to A). At 10 μM of Sph the effect was more evident (Fig. 4C). Finally, at 20 μM, all the parasites were rounded (Fig. 4D). We also observed that, as the concentration of the sphingolipid was increased, the parasites lost its mobility. Even more, we could observe that parasites changes in the shape and mobility were followed by cell death, as assessed with the use of trypan blue exclusion.

4. Discussion

Ca^{2+} is known to exert important functions in the human parasite *L. mexicana* [1]. However, the mechanism of Ca^{2+} entry in these parasites is unknown. In the present work we show compelling evidences for the presence of a plasma membrane Ca^{2+} channel in *L. mexicana,* with characteristics that resemble the human L-type VGCC. Thus, the channel is blocked by well-known L-type VGCC antagonists, such as verapamil, nifedipine and diltiazem. Even more, this channel can be opened by BayK 8644, a very specific L-type VGCC agonist.

A very interesting finding obtained in the course of this work was that Sph was able to activate the *L. mexicana* plasma membrane Ca^{2+} channel. This sphingolipid is known to inhibit many human enzymes, such as protein kinase C [21] and several Ca^{2+}-calmodulin dependent enzymes [22]. Concerning Ca^{2+} movements, it has been demonstrated that Sph inhibits the human plasma membrane Ca^{2+}-ATPase, PMCA, [23] and is also able to open a TRP channel [24]. Sph is also known to increase the intracellular Ca^{2+} concentration in several cells, such as Jurkat T lymphocytes [18], by a yet unknown mechanism. However, there is no report of an action of Sph in *Leishmania* parasites. Nevertheless, the presence of this and other sphingolipids in these parasites is well documented [16,17]. Experiments conducted with null-mutants of key enzymes from the sphingolipids pathway in *L. major* indicate that sphingolipids are not relevant, at least to support cell growth of promastigotes in culture [16]. Instead, they seem to be necessary for the differentiation to amastigotes and for the production of acidocalcisomes [17,25]. Origin of these neutral sphingolipids is not entirely known. There are even doubts if they are synthesized by the parasites or salvaged from the mammalian host [17–25].

L-type Ca^{2+} channels are found in many excitable cell types, including muscle, neuronal, and endocrine cells, where they initiate Ca^{2+}-dependent responses such as contraction and secretion.

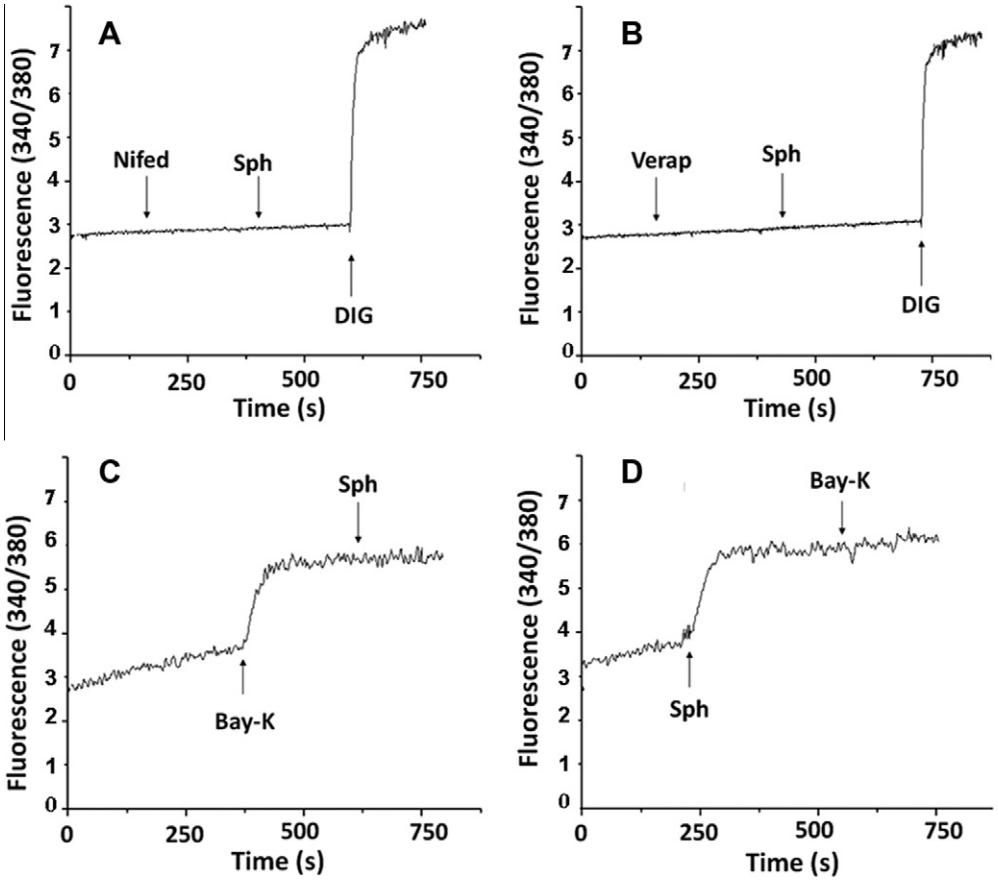

Fig. 2. Effect of Ca^{2+} channel blockers (Nifedipine and Verapamil) and Ca^{2+} channel agonist (BayK 8644) on the intracellular Ca^{2+} concentration of *L. mexicana*. Promastigotes of *L. mexicana* were loaded with Fura 2, as explained under "Experimentals". (A) Nifedipine (Nifed, 2 μM) and then Sphingosine (Sph, 20 μM) were added (arrow) directly to the stirring cuvette in the presence of 2 mM $CaCl_2$. (B) Verapamil (Verap, 4 μM) and then Sphingosine (Sph, 20 μM) were added (arrows) directly to the stirring cuvette in the presence of 2 mM $CaCl_2$. (C) BayK 8644 (BayK, 2 μM) and then Sphingosine (Sph, 20 μM) were added (arrows) directly to the stirring cuvette, in the presence of 2 mM $CaCl_2$ (D) Sphingosine (Sph, 20 μM) and then BayK 8644 (BayK, 2 μM) and were added (arrows) directly to the stirring cuvette in the presence of 2 mM $CaCl_2$. Traces are representative of at least four independent experiments.

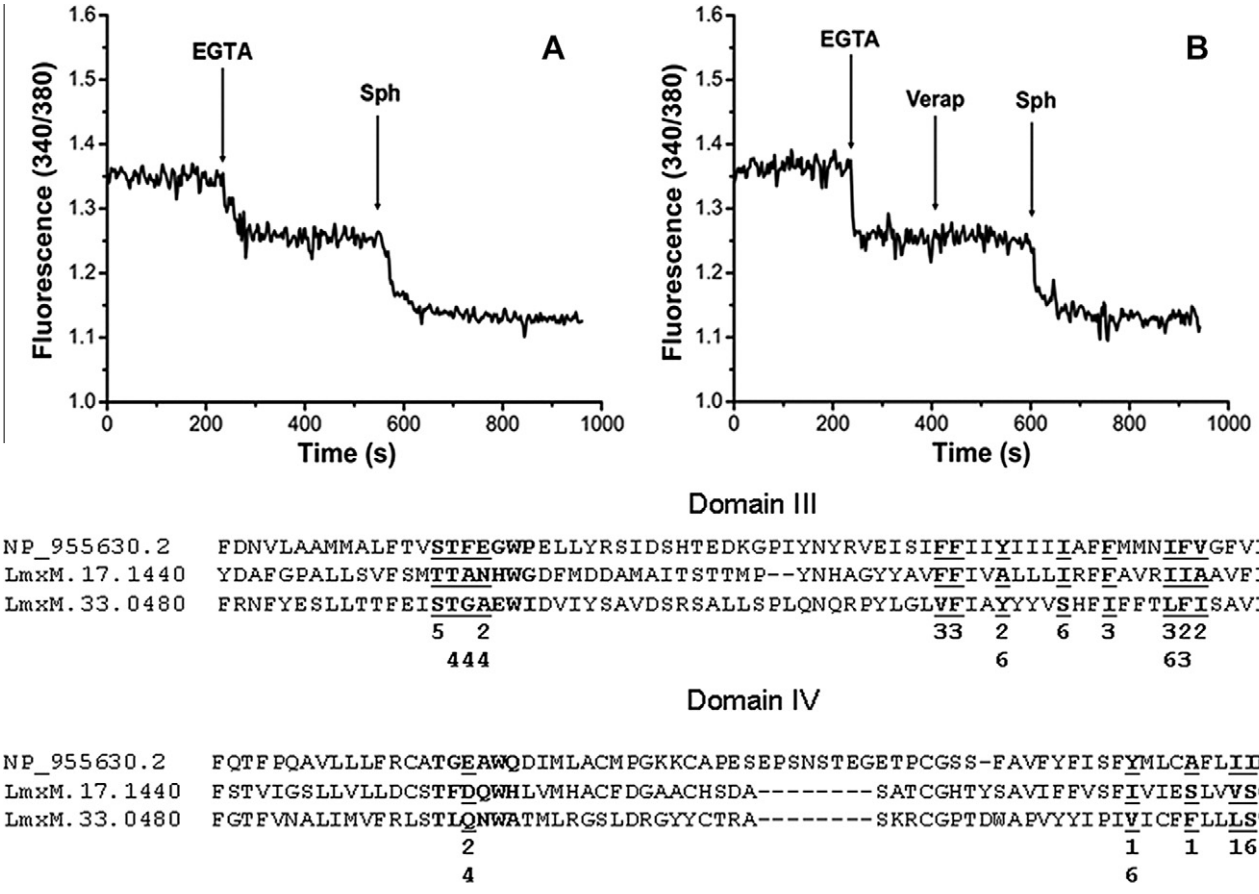

Fig. 3. (Top) Effect of Sphingosine and Verapamil in the absence of extracellular Ca^{2+} on the intracellular Ca^{2+} concentration of *L. mexicana* Top: Promastigotes of *L. mexicana* were loaded with Fura 2, as explained under "Experimentals". (A) EGTA (2 mM) and then Sphingosine (Sph, 20 μM) were added (arrows) directly to the stirring cuvette. (B) EGTA (2 mM) and then Verapamil (Verap, 4 μM) and Sphingosine (Sph, 20 μM) were added (arrows) directly to the stirring cuvette. Traces are representative of at least four independent experiments. (Bottom) Sequence alignments of the IIIS6 and IVS6 domains of Cav channel with *Leishmania* channel homologes. The amino acid sequences next to the selectivity filter are in bold, and the amino acids associated with DHP or PAA sensibility are in bold and underlined. References associated with sensitivity to DHP or PAA are indicated under each animo acid using the next legend: (1,2) Hockerman et al., 1997a,b; (3) Hering et al. (1997); (4) Dilmac et al., 2004; in reference to PAA and (5) Yagamaguchi et al. (2000), (6) Lipkind and Fozzard, 2003, in reference to DHP.

The pore-forming α1 subunits of voltage-gated Ca^{2+} channels consist of four homologous domains (I–IV), each containing six putative transmembrane segments (S1–S6). L-type channels share a common pharmacological profile, including high-affinity voltage- and frequency-dependent block by dihydropyridines (DHP) as nifedipine, phenylalkylamines (verapamil), and benzodiazepines (diltiazem). These drugs are thought to bind to three separate receptor sites on L-type Ca^{2+} channels that are allosterically linked.

L. mexicana Ca^{2+} channel sequences were searched into genome using the BLASTP search tool of GENEDB (http://www.genedb.org/blast/submitblast/GeneDB_Lmexicana). BLASTP analysis was carried out using the human sequence of the L-Type VGCC α1C subunit (Gen bank accession number NP_955630.2) (hCav). Two sequences, annotated LmxM.17.1440 and LmxM.33.0480 were identified as possible Ca^{2+} channel and the presence of conserved domains was confirmed using Pfam and InterProScan tools (see Fig. 3, bottom). Sequences were selected because they produced significant alignments with E-values better than threshold. LmxM.17.1440 polypeptide contains 1414 amino acids, and theoretically it has a molecular mass of 154.400, and LmxM.33.0480 polypeptide has 2547 amino acids and a molecular mass of 290.300, while hCav has 2221 amino acids and a molecular mass of 248.977. Prole and Taylor [14] identified in kinetoplastids some Cav channel homologues. These channels are predicted to possess 18–24 transmembrane domains consistent with a four-domain structure formed from a single subunit, similar to the organization of mammalian Cav channels. No physiological evidence has been presented about these channels to date, and the signal that induces Ca^{2+} influx remains unknown. It is also uncertain if they are sensitive to phenylalkylamines, benzodiazepines or dihydropyridines. However, Tempone et al. [15] reported the antileishmanial activity of DHP against cutaneous and visceral species of Leishmania. Our results revealed for the first time that sphingosine induced a Ca^{2+} signal in *L. mexicana*. The signal was inhibited by verapamil, nifedipine and diltiazem, and was activated by BayK 8644, the classical L-type VGCC inhibitors and activator, respectively. Results from different experimental approaches implicate the IIIS5, IIIS6, and IVS6 transmembrane segments of the α1 subunits of L-type Ca^{2+} channels in the binding of all four classes of drugs [26–29]. The alignment of human Cav α1C, LmxM.17.1440 and LmxM.33.0480 was made using ClustalW 2 (European Bioinformatics Institute) (Fig. 3, bottom). This alignment revealed that many residues in III S6 and IV S6 domain associated with DHP or phenylalkylamines sensitivity are conserved or have conservative changes in the Leishmania sequences.

For example, Yamaguchi et al. [29] demonstrated that the hydroxyl group of Ser1115 in IIIS5–S6 linker of the L-type Ca^{2+} channel α1C subunit plays a critical role in DHP binding and in the action of DHP Ca^{2+} channel agonists. This residue is located next to the homologous domain that composes the selectivity filter into the segment IIIS6. LmxM.33.0480 sequence has conserved this serine residue. In the case of LmxM.17.1440 sequence, this position is

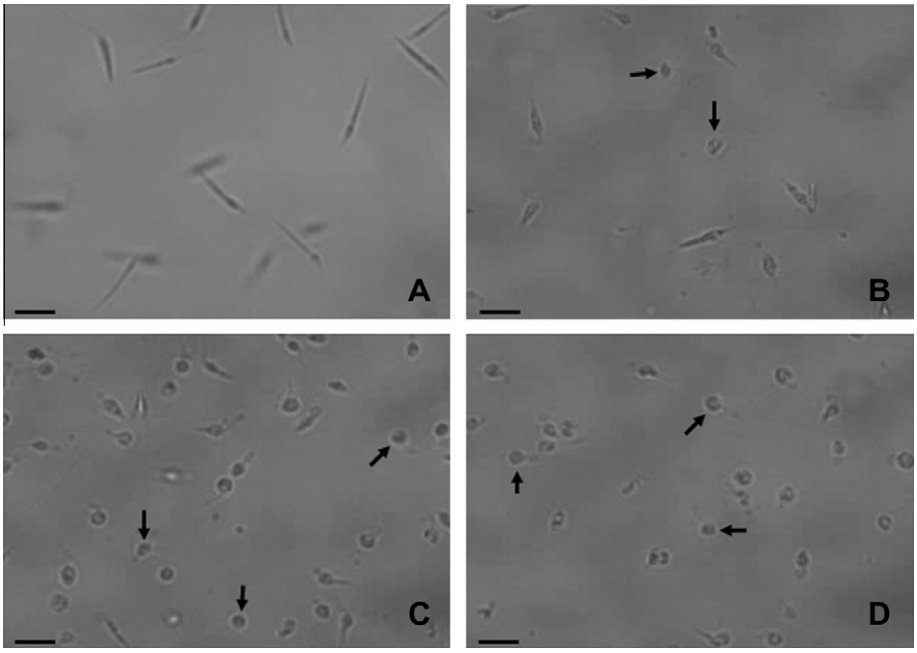

Fig. 4. Effect of Sphingosine on promastigotes of *L. mexicana*. Sphingosine was added to cultured promastigotes at the indicated concentration, and after 20 min the different photographs were taken using an 100X immersion objective and phase contrast microscopy. A, Control. B, Sph, 5 μM. C, Sph, 10 μM. D, Sph, 20 μM. Bars in images represent 10 μm.

occupied by a threonine residue, a conservative substitution. The amino acid similarities could explain the leishmanial sensitivity to DHP and phenylalkylamines.

The dramatic and relatively rapid effect of Sph on the shape and mobility, leading to the death of the parasites, was unexpected and deserves a special mention. These is not due merely to the Ca^{2+} increase induced by sphingosine since a rapid Ca^{2+} augment do not induce the parasite death [30]. Nevertheless, as a naturally occurring mechanism, it is conceivable that the production of Sph by the cell in a particular moment should be rapid, local and transitory. This is not the case under the experimental conditions set in this work, since Sph was not removed at any moment. It is not even known if the effect of Sph is triggered by direct binding to the channel or through an enzymatic cascade involving kinases or other protein modulators. These experiments, together with electrophysiological studies (i.e. voltage dependency), remain to be done, in order to further characterize this interesting channel.

Acknowledgments

We thank Dr. Lourdes Plaza for critically reviewing the manuscript. This work was supported by grants from Fondo Nacional de Ciencia, Tecnologìa e Investigación, Venezuela (FONACIT) (No. 2011000884), and from the Consejo de Desarrollo Científico y Humanístico (C.D.C.H.-U.C.V.), Universidad Central de Venezuela to G.B.

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