



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

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### ABSTRACT

The disruption of the intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  entry to these cells. Here we show the presence of a  $\text{Ca}^{2+}$  channel in the plasma membrane of these parasites. This channel has many characteristics similar to the human L-type voltage-gated  $\text{Ca}^{2+}$  channel. Thus,  $\text{Ca}^{2+}$  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.

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### 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  $\text{Ca}^{2+}$  is involved in several functions in these parasites [2], including differentiation and host cell invasion [3,4]. The mechanisms involved in the intracellular  $\text{Ca}^{2+}$  regulation in these parasites are quite well described [1–3]. They possess a distinctive single mitochondria able to accumulate  $\text{Ca}^{2+}$  by an electrophoretic uniporter, using as driving force for  $\text{Ca}^{2+}$  uptake, the mitochondrial electrochemical potential [5]. These parasites also possess acidocalcisomes, interesting acidic organelles able to accumulate large amounts of  $\text{Ca}^{2+}$  [6]. Acidocalcisomes are very relevant concerning the bioenergetic of these parasites since they accumulate  $\text{Ca}^{2+}$  in combination with pyrophosphate, which is an energy source, alternative to ATP [2,3]. The endoplasmic reticulum is also involved in  $\text{Ca}^{2+}$  uptake [2,3]. At the plasma membrane, the parasite is able to extrude  $\text{Ca}^{2+}$ , due to the presence

of a  $\text{Ca}^{2+}$ -ATPase [7]. However, little is known concerning the mechanisms of  $\text{Ca}^{2+}$  entry. It has been recently shown that many drugs of current use against this parasite exert their action through the disruption of its intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  homeostasis, affecting the acidocalcisomes, and also collapsing the electrochemical mitochondrial potential, thus inducing the release of  $\text{Ca}^{2+}$  to the cytoplasm [9,11,12].

Concerning the mechanisms of  $\text{Ca}^{2+}$  entry to these parasites, the presence of a  $\text{Ca}^{2+}$  channel in the plasma membrane should be warranted since these parasites are able to significantly change the intracellular  $\text{Ca}^{2+}$  content depending on different conditions, for example, during cell invasion [4]. However, information related to the presence of such a  $\text{Ca}^{2+}$  channel is scarce. It has been reported that arachidonic acid induces an intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  [13]. Melittin, on the other hand, is able to induce a  $\text{Ca}^{2+}$  influx, which appears to depend on the activity of a  $\text{PLD}_2$ . 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  $\text{Ca}^{2+}$  channel (VGCC) could be present in

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these parasites [14]. In the present work we demonstrate the presence of a  $\text{Ca}^{2+}$  channel in the plasma membrane of *L. mexicana*, with many similarities with the human VGCC. Accordingly, nifedipine and verapamil, classical inhibitors of this channel, known to affect the growth of *Leishmania* sp. [15], block the parasite  $\text{Ca}^{2+}$  channel. However, differently from its putative human counterpart, the *L. mexicana*  $\text{Ca}^{2+}$  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 $\text{Ca}^{2+}$ measurements

Promastigotes were loaded with the fluorescent ratiometric  $\text{Ca}^{2+}$  indicator Fura 2, to estimate variations on intracellular  $\text{Ca}^{2+}$  concentration, as described [9]. Briefly,  $2 \times 10^8$  parasites were collected by centrifugation at 600g for 2 min and washed twice in PBS buffer plus 1% glucose. Then, the parasites were loaded with Fura 2-AM (6  $\mu\text{M}$ ), probenecid (12  $\mu\text{M}$ ) and pluronic acid (12  $\mu\text{M}$ ) 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  $\lambda$  Em at the two excitation wavelength.

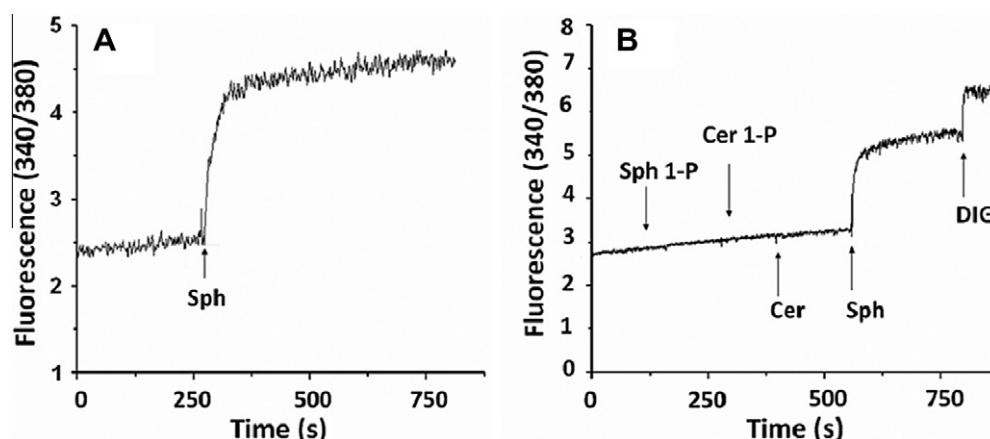
## 3. Results

Different sphingolipids, such as ceramide, sphingosine (Sph), ceramide-1-P and sphingosine-1-P, are known to increase the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) 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  $\text{Ca}^{2+}$  indicator Fura 2. In Fig. 1A it can be observed that addition of 20  $\mu\text{M}$  Sph, concentration reported to exert its optimal effect on the  $[\text{Ca}^{2+}]_i$  in human cell lines [18], is able to induce a large increase in the  $[\text{Ca}^{2+}]_i$  of these parasites. Lower concentrations of Sph also increase the  $[\text{Ca}^{2+}]_i$  of these parasites, but to a lesser extent in a dose-dependent manner (results not shown). For this reason we used 20  $\mu\text{M}$  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  $[\text{Ca}^{2+}]_i$  in human cell lines [18,19], were not able to affect the intracellular  $\text{Ca}^{2+}$  content of these parasites (Fig. 1B). Digitonin was added in these experiments to allow the maximal  $\text{Ca}^{2+}$  entry to the cells. Since digitonin was able to further induce an increase in the  $\text{Ca}^{2+}$  fluorescence, the  $\text{Ca}^{2+}$  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  $[\text{Ca}^{2+}]_i$ , we added sequentially the different inhibitors prior to the addition of the sphingolipid. In Fig. 2A and B it can be observed that nifedipine and verapamil, at concentration used to block the human VGCC, totally blocked the effect of Sph. Diltiazem (10  $\mu\text{M}$ ) 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $[\text{Ca}^{2+}]_i$  increase, produced a dramatic fall. This result is compatible with the exit of the basal intracellular  $\text{Ca}^{2+}$  to the outer medium,



**Fig. 1.** Effect of different sphingolipids on the intracellular  $\text{Ca}^{2+}$  concentration of *L. mexicana*. Promastigotes of *L. mexicana* were loaded with Fura 2, as explained under “Experimentals”. (A) Sphingosine (20  $\mu\text{M}$ ) was added (arrow) directly to the stirring cuvette in the presence of 2 mM  $\text{CaCl}_2$ . (B) Sphingosine 1-P (Sph 1-P, 20  $\mu\text{M}$ ), Ceramide 1-P (Cer 1-P, 20  $\mu\text{M}$ ), Ceramide (Cer, 20  $\mu\text{M}$ ), Sphingosine (Sph, 20  $\mu\text{M}$ ) and Digitonin (DIG, 30  $\mu\text{M}$ ) were added (arrows) directly to the stirring cuvette in the presence of 2 mM  $\text{CaCl}_2$ . 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  $\text{Ca}^{2+}$  fluxes in both directions, similar to its human analog. In order to study if verapamil could block this  $\text{Ca}^{2+}$  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  $\mu\text{M}$  of the sphingolipid to promastigotes of *L. mexicana*, the parasites started to take a rounded form (compare Fig. 4B to A). At 10  $\mu\text{M}$  of Sph the effect was more evident (Fig. 4C). Finally, at 20  $\mu\text{M}$ , 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.

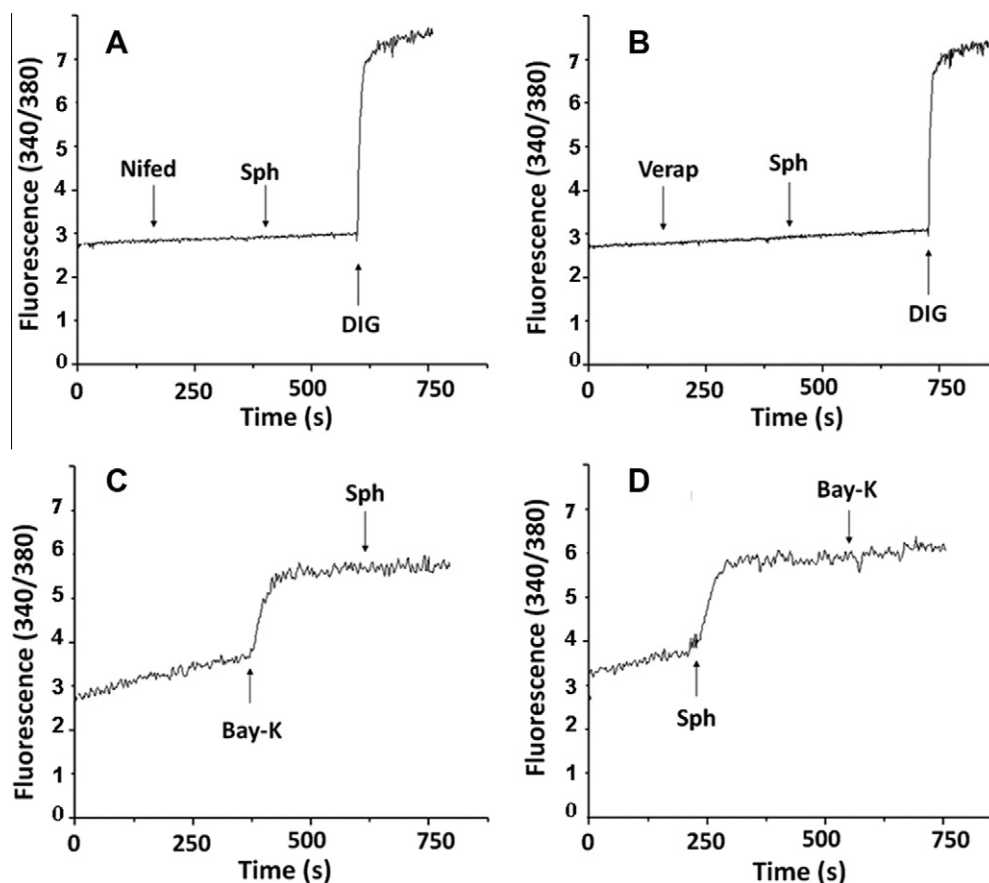
#### 4. Discussion

$\text{Ca}^{2+}$  is known to exert important functions in the human parasite *L. mexicana* [1]. However, the mechanism of  $\text{Ca}^{2+}$  entry in these parasites is unknown. In the present work we show compelling evidences for the presence of a plasma membrane  $\text{Ca}^{2+}$  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.

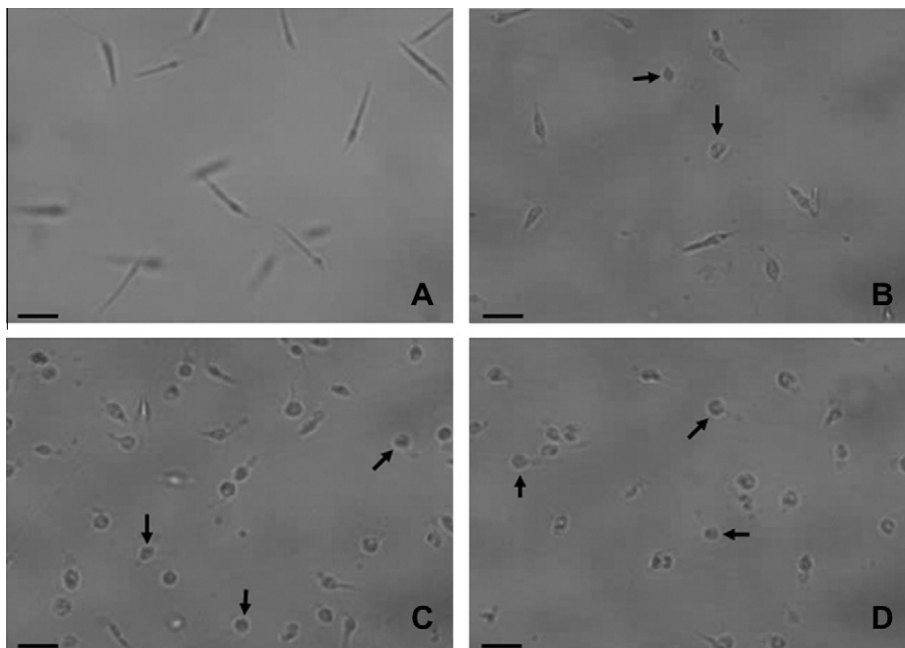
A very interesting finding obtained in the course of this work was that Sph was able to activate the *L. mexicana* plasma membrane  $\text{Ca}^{2+}$  channel. This sphingolipid is known to inhibit many human enzymes, such as protein kinase C [21] and several  $\text{Ca}^{2+}$ -calmodulin dependent enzymes [22]. Concerning  $\text{Ca}^{2+}$  movements, it has been demonstrated that Sph inhibits the human plasma membrane  $\text{Ca}^{2+}$ -ATPase, PMCA, [23] and is also able to open a TRP channel [24]. Sph is also known to increase the intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channels are found in many excitable cell types, including muscle, neuronal, and endocrine cells, where they initiate  $\text{Ca}^{2+}$ -dependent responses such as contraction and secretion.



**Fig. 2.** Effect of  $\text{Ca}^{2+}$  channel blockers (Nifedipine and Verapamil) and  $\text{Ca}^{2+}$  channel agonist (BayK 8644) on the intracellular  $\text{Ca}^{2+}$  concentration of *L. mexicana*. Promastigotes of *L. mexicana* were loaded with Fura 2, as explained under “Experimental”. (A) Nifedipine (Nifed, 2  $\mu\text{M}$ ) and then Sphingosine (Sph, 20  $\mu\text{M}$ ) were added (arrows) directly to the stirring cuvette in the presence of 2 mM  $\text{CaCl}_2$ . (B) Verapamil (Verap, 4  $\mu\text{M}$ ) and then Sphingosine (Sph, 20  $\mu\text{M}$ ) were added (arrows) directly to the stirring cuvette in the presence of 2 mM  $\text{CaCl}_2$ . (C) BayK 8644 (BayK, 2  $\mu\text{M}$ ) and then Sphingosine (Sph, 20  $\mu\text{M}$ ) were added (arrows) directly to the stirring cuvette, in the presence of 2 mM  $\text{CaCl}_2$ . (D) Sphingosine (Sph, 20  $\mu\text{M}$ ) and then BayK 8644 (BayK, 2  $\mu\text{M}$ ) were added (arrows) directly to the stirring cuvette in the presence of 2 mM  $\text{CaCl}_2$ . Traces are representative of at least four independent experiments.





**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  $\mu$ M. C, Sph, 10  $\mu$ M. D, Sph, 20  $\mu$ M. Bars in images represent 10  $\mu$ m.

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  $\text{Ca}^{2+}$  increase induced by sphingosine since a rapid  $\text{Ca}^{2+}$  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.

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