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

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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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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,

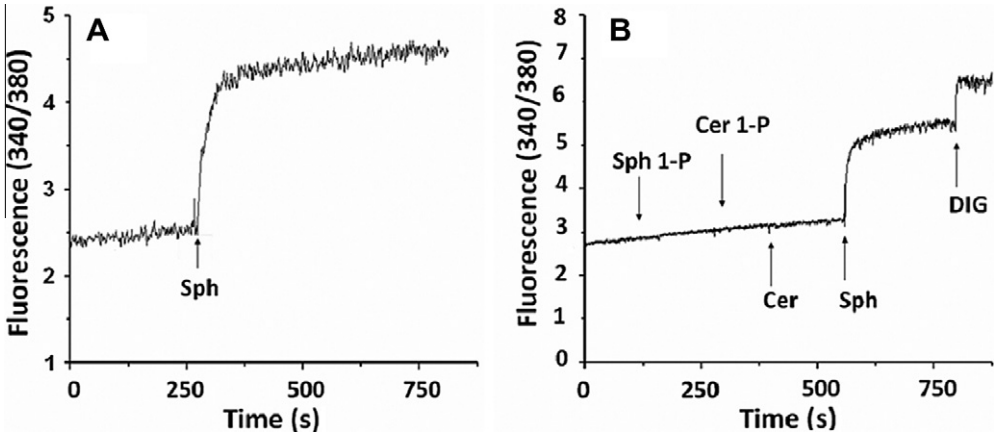

Fig. 1. Effect of different sphingolipids on the intracellular Ca^{2+} concentration of *L. mexicana*. Promastigotes of *L. mexicana* were loaded with Fura 2, as explained under "Experimentals". (A) Sphingosine (20 μM) was added (arrow) directly to the stirring cuvette in the presence of 2 mM $CaCl_2$. (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.