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Ca^{2+}-activated transbilayer movement of plasma membrane phospholipids in *Leishmania donovani* during ionomycin or thapsigargin stimulation

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ABSTRACT

The protozoan parasite *Leishmania* causes serious infections in humans all over the world. After being inoculated into the skin through the bite of an infected sandfly, *Leishmania* promastigotes must gain entry into macrophages to initiate a successful infection. Specific, surface exposed phospholipids have been implicated in *Leishmania*–macrophage interaction but the mechanisms controlling and regulating the plasma membrane lipid distribution remains to be elucidated. Here, we provide evidence for Ca^{2+}-induced phospholipid scrambling in the plasma membrane of *Leishmania donovani*. Stimulation of parasites with ionomycin increases intracellular Ca^{2+} levels and triggers exposure of phosphatidylethanolamine at the cell surface. We found that increasing intracellular Ca^{2+} levels with ionomycin or thapsigargin induces rapid transbilayer movement of NBD-labelled phospholipids in the parasite plasma membrane that is bidirectional, independent of cellular ATP and not specific to the polar lipid head group. The findings suggest the presence of a Ca^{2+}-dependent lipid scramblase activity in *Leishmania* parasites. Our studies further show that lipid scrambling is not activated by rapid exposure of promastigotes to higher physiological temperature that increases intracellular Ca^{2+} levels.

1. Introduction

Phospholipid flip-flop in the plasma membrane of eukaryotic cells is highly regulated so that cells are able to maintain a non-random lipid distribution between the two leaflets of the plasma membrane. In general, the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are largely confined to the cytosolic leaflet and sphingolipids (i.e. sphingomyelin and glycosphingolipids) are enriched in the exoplasmic leaflet [1,2]. This lipid asymmetry is thought to rely on active transport of specific lipids across the bilayer by the action of energy-dependent translocases [3,4]. Prime candidates for outward-directed lipid translocases are members of the ATP binding cassette (ABC) transporter family [5], whereas inward-directed lipid translocases belong to the P4 subfamily of P-type ATPases [6–8]. In addition to these energy-dependent translocases, certain eukaryotic cells contain a phospholipid scramblase, a putative membrane protein that upon activation facilitates a rapid bidirectional movement of phospholipids between the two plasma membrane leaflets [9]. The scramblase activity is typically, but not always [10], activated by elevation of cytoplasmic calcium concentration and disrupts the lipid asymmetry set up by the ATP-dependent translocases [11–13].

Dissipation of lipid asymmetry and externalization of aminophospholipids play a prominent role during cell fusion, activation of the coagulation cascade, virus entry into host cells, and the recognition and phagocytic clearance of apoptotic cells [14–16]. Loss of plasma membrane lipid asymmetry has also been implicated in the infectivity of *Leishmania*, an obligate, intracellular parasite of humans and other mammals that infect cells of the mononuclear phagocyte lineage. The parasite has a digenic life cycle, residing as flagellated extracellular promastigote in the gut of the insect vector and as obligatory intracellular aflagellated amastigote found in the parasitophorous vacuoles of mammalian macrophages. The observation of annexin V binding on the cell surface of *Leishmania* parasites led to the hypothesis that the protozoan parasite might take advantage of a regulated loss of lipid asymmetry and PS presentation to invade and survive in host macrophages [17–20].

Little is known about the regulation of the membrane lipid organisation in *Leishmania*. Recent studies have provided

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; PI, propidium iodide; NBD, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); PLSCR, phospholipid scramblase.

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evidence for the presence of ATP-dependent lipid translocases in the *Leishmania* plasma membrane [21–24]. A prime candidate for the inward-directed translocase is a complex formed by the miltefosine transporter LdMT, which belongs to the P4 subfamily of P-type ATPases, and LdRos3, a noncatalytic subunit of LdMT related to the Cdc50 family. Loss of either LdMT or LdRos3 essentially abolishes the ATP-dependent inward translocation of fluorescent 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labelled analogues of PE and phosphatidylcholine (PC) across the plasma membrane and causes aberrant exposure of endogenous PE at the parasite cell surface [24]. Furthermore, a number of ABC transporters appear to facilitate an outward-directed lipid transport. *Leishmania* ABCB1-like transporter reduced the accumulation of a fluorescent lipid analogue of PC [25]. Likewise, *Leishmania* ABC transporters related to the human ABCA and ABCG family have been linked to active outward lipid transport of PC, PE and PS [26–29].

Here, we investigated the phospholipid distribution and dynamics in the plasma membrane of *Leishmania donovani* upon elevation of intracellular calcium levels with ionomycin, thapsigargin or upward temperature shifts. Our results indicate that *Leishmania* parasites possess Ca^{2+}-activated lipid scrambling.

2. Materials and methods

2.1. Cells and ATP depletion

Promastigotes of *L. donovani* (MHOM/IN/80/DD8 and MHOM/ET/67/HU3) and *LdMT−/−* (kindly provided by Santiago Castanys and Francisco Gamarro, Instituto de Parasitología y Biomedicina, Granada, Spain) were grown at 26 °C in M-199 medium (Invitrogen) supplemented with 40 mM HEPES, 100 μM adenosine, 500 ng/ml hemin, 10 μM 6-biopterin and 10% heat-inactivated fetal calf serum (Gibco). THP-1 cells were kindly provided by Juan Patron and cultured at 37 °C with 5% CO_2 in RPMI-1640 medium (Gibco) supplemented with 2 mM glucose, 50 μM β-mercaptoethanol, 1 mM Na-pyruvate, and 10% fetal calf serum. Unless indicated otherwise, all materials were purchased from Sigma–Aldrich. For ATP depletion, parasites were incubated for 30 min at 28 °C in glucose-free HPMI containing 5 mM of 2-deoxyglucose, 50 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 5 mM sodium azide. ATP levels were determined in a BioOrbit-Luminometer using a luciferin–luciferase assay (Colora, Lorch, Germany) following the manufacturer's instructions.

2.2. Measurement of cytosolic Ca^{2+}

Log phase promastigotes (10^7/ml) in supplemented M-199 medium without FCS were loaded for 40 min at 25 °C with 5 μM Fluo-4/AM (from a 10 mM DMSO stock; Invitrogen). After washing in supplemented M-199 medium, parasites were suspended in HPMI (132 mM NaCl, 3.5 mM KCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, 1 mM sodium pyruvate, 20 mM HEPES, pH 7.4) to a concentration of 10^7/ml and stimulated with 20 μM ionomycin (from a 1.4 mM DMSO stock, Ascent Scientific, UK), 10 μM thapsigargin (from a 2 mM DMSO stock; Invitrogen) or left untreated. At indicated time points, samples were analyzed by confocal microscopy and flow cytometry.

2.3. Analysis of transbilayer NBD-lipid movement

Palmitoyl-(NBD-hexanoyl)-phosphatidylcholine (NBD-PC), palmitoyl-(NBD-hexanoyl)-phosphatidylethanolamine (NBD-PE), palmitoyl-(NBD-hexanoyl)-phosphatidylserine (NBD-PS) and 6-NBD-hexanoyl-sphingosine-1-phosphocholine (NBD-sphingomyelin; NBD-SM) were purchased from Avanti Polar Lipids (Birmingham, AL). Appropriate amounts of analogues (5 nmol of NBD-lipids for 10^7 cells) in chloroform/methanol (1:1) were transferred to a glass tube, dried under nitrogen, dissolved in 5 μl absolute ethanol. Log phase promastigotes were harvested by centrifugation (1000 × *g*, 10 min), washed twice with HPMI. To block the conversion of NBD-lipids by phospolipases, the cell suspension (10^7 parasites/ml) was pre-incubated with 5 μM 3-(4-octadecyl)-benzoylacrylic acid (Biomol, Hamburg, Germany) and 1 mM phenylmethanesulphonylfluoride for 30 min at 25 °C. For calcium depletion, parasites (10^6 cells) were incubated for 30 min at 25 °C in M199 medium containing 0.05–0.1 mM BAPTA-AM (Invitrogen), washed twice and suspended in HPMI without calcium but supplemented with 2 mM ethylene glycol tetraacetic acid (EGTA). Stimulation of parasites with 20 μM ionomycin was performed for 30 min at 25 °C. Unless otherwise indicated, parasites were then shifted to 4 °C and labelled with 5 μM NBD-lipid. At indicated time points, samples were removed and analyzed by confocal microscopy and flow cytometry as described before [24], except that parasites were examined without prior washing with albumin-containing medium. In this way, the total fluorescence associated with the parasites at a given time point was determined.

2.4. Analysis of NBD-lipid metabolism

Lipids were extracted from cells and medium as described [30] and separated by thin-layer chromatography using chloroform/methanol/water (65:25:4, v/v/v). NBD-lipid standards were chromatographed on the same plate. Fluorescent lipid spots were quantified on a FLA-3000 Fuji Imaging System (Raytest, Straubenhardt, Germany) equipped with a 488 nm laser and a 515 nm long pass emission filter. Image analysis was performed using Aida Image Analyser 3.24 software (Raytest, Straubenhardt, Germany).

2.5. Biotin-Ro and annexin V assay

To visualize endogenous PE on the cell surface, ionomycin-stimulated or untreated promastigotes (5×10^6) were incubated in 20 μl HPMI containing 38 μM biotinylated Ro09-0198 (provided by Kazuma Tanaka). After 1 h at 2 °C, cells were washed with phosphate buffered saline (PBS) containing 0.5% (w/v) bovine serum albumin and then fixed with PBS containing 5% (w/v) formaldehyde for 1 h at 30 °C. Promastigotes were then washed in PBS, suspended in 250 μl PBS containing 5 μg/ml Streptavidin-FITC and incubated for 30 min at 25 °C prior to microscopy analysis. To measure exposure of endogenous PS on the cell surface, about 5×10^5 parasites were incubated on ice for 10 min at 2 °C in the dark with 125 ng annexin V-FITC and 1 μg PI in 0.5 ml of binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Cells were washed, suspended in 0.5 ml of binding buffer and subjected to microscopy.

2.6. Fluorescence microscopy

Confocal laser scanning microscopy was performed using an inverted Fluoview 1000 microscope (Olympus, Tokio, Japan) and a 60× (N.A. 1.35) oil-immersion objective. Fluorescence of FITC and CellTracker Green was excited with a 488 nm argon laser and recorded between 500 and 530 nm. Fluorescence of propidium iodide and CellTracker CM-Dil was excited with a 559 nm argon laser and recorded between 570 and 600 nm. Images with a frame size of 256 × 256 pixels were acquired.

2.7. Flow cytometry

Flow cytometry analysis was performed on a Becton Dickinson FACS (San Jose, CA) equipped with an argon laser (488 nm) using Cell Quest software. One microlitre of 1 mg/ml PI in H_2O was added to 200 μl cell suspension just before analysis. Ten thousand