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Rapid transport of phospholipids across the plasma membrane of *Leishmania infantum*☆

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Abstract

The internalization of fluorescent phospholipid analogs of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) in *Leishmania infantum* promastigotes was studied. We observed a rapid inward redistribution of NBD-PC, -PE, and -PS across the plasma membrane at 28 and 4 °C. This internalization was shown to be independent of the endocytic activity of parasites. Rapid inward movement was coupled to an energy-dependent transporter because it was almost inhibited by depletion of cellular ATP and was blocked after pretreatment with *N*-ethylmaleimide (NEM). In contrast, NBD-SM traverses the plasma membrane by passive flip-flop. By comparing this pattern of phospholipid transbilayer movement with those known from other eukaryotic cells, candidate lipid transporters are discussed.

Keywords: Parasites; Leishmania; Lipid translocation; NBD; Phospholipid

Although the life cycle of the protozoan parasite *Leishmania* is known, essential processes and the molecular basis of the infection by the parasite remain to be elucidated. It has been suggested that parasite lipid organization could resemble an essential determinant for being phagocytosed by macrophages in order to survive in the host organism. Exposure of phosphatidylserine on the exoplasmic leaflet of the plasma membrane is involved in amastigotes infection of host cells and inhibits the antileishmanial activity of macrophages [1]. Furthermore, phospholipid analogs such as edelfosine (alkyl-glycerophosphocholines) and miltefosine (alkylphosphocholine) exhibit a significant antileishmanial activity. Although the relevance of lipids for infection by parasites is recognized, nothing is known about transbilayer movement and organization of lipids in the plasma membrane of *Leishmania*. In this study we investigate the internalization of fluorescent phospholipid analogs by the promastigote form of *Leishmania infantum*. We found a rapid protein and energy dependent inward movement for NBD-PC, -PE, and -PS across the plasma membrane of *L. infantum*. In contrast, NBD-SM only traverses the plasma membrane via passive diffusion.

Materials and methods

Leishmania strain and culture. Late- and early-log phase promastigotes of *L. infantum* (strain 21578, LEM 2592, Montpellier, France) were grown at 28 °C in modified RPMI-1640 medium (Gibco BRL) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Gibco BRL). If not stated otherwise, late-log promastigotes were used. Promastigotes were harvested by centrifugation (2000*g*, 8 min), washed twice with phosphate-buffered saline (PBS; 130 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4), and resuspended at 2×10^5 parasites/ml in HPMI (132 mM NaCl, 3.5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 20 mM Hepes, pH 7.2). HPMI was routinely supplemented with 5 mM DFP to block the catabolism of NBD-lipids.

☆ *Abbreviations:* APLT, aminophospholipid translocase; DFP, di-isopropyl fluorophosphate; MDR1 Pgp, multidrug resistance 1 P-glycoprotein; MRP1, multidrug resistance related protein 1; NBD, [*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

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ATP depletion. By incubating cells for 30 min at 28 °C in glucose-free HPMI containing either 5 mM of 2-deoxyglucose and 20 mM sodium azide, or 20 mM sodium azide, the intracellular ATP level was reduced to 5% and 20% of that in control cells, respectively, as determined by a luciferin–luciferase assay (Colora, Lorch, Germany).

Labeling with NBD-lipids. 1-Palmitoyl-(NBD-hexanoyl)-glycerophospholipids and NBD-sphingomyelin (NBD-SM) were from Avanti Polar Lipids (Birmingham, AL). Synthesis of 1-octadecanoxy-(NBD-hexanoxy)-PC (diether NBD-PC) was described previously [2]. Appropriate amounts of NBD-lipids dissolved in chloroform were transferred to a glass tube, dried under nitrogen, solubilized in 5 μl absolute ethanol, and resuspended in 200 μl HPMI with vortexing to yield a 10 μM suspension. Labeling was initiated by adding 80 μl ice-cold labeled suspension to 1170 μl cell suspension at 4 °C. After 5 min of incubation on ice, non-inserted analogs were removed by washing the cells with ice-cold HPMI. Subsequently, cells were incubated at 4 or 28 °C for various times before further analysis. The amount of NBD-lipid added to the cells corresponded to about 0.25 mol% of the total cell phospholipid concentration. Total phospholipid was quantified after lipid extraction by phosphate determination [3]. Promastigotes contained about 20 nmol phospholipid per 15×10^6 cells (6 determinations).

Fluorescence spectrometry. The amount of NBD-lipids in the exoplasmic leaflet was measured using the dithionite assay. Briefly, 250 μl aliquots of the labeled cell suspension were diluted in 1550 μl HPMI precooled at 4 °C. The fluorescence intensity was measured at 540 nm (excitation 470 nm) with a SLM-AMINCO fluorescence spectrometer (AMICO-Bowman, Urbana, IL). After 1 min, 18 μl of 1 M sodium dithionite (freshly dissolved in 100 mM Tris–HCl, pH 10) was added. Dithionite rapidly destroyed the fluorescence of the NBD-lipids in the outer leaflet of the plasma membrane. The fluorescence plateau reached reflects NBD-lipids inside the cells. To make all analogs accessible to dithionite, Triton X-100 was added at a final concentration of 0.5%. The relative amount of NBD-lipids protected against dithionite was calculated according to [4].

Analysis of NBD-lipid metabolism. Lipids were extracted from cells and medium as described [5] and separated by thin-layer chromatography using chloroform/methanol/water (65:25:4, v/v). 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).

Flow cytometry. Flow cytometry was performed on a Becton–Dickinson FACS (San Jose, CA) equipped with an argon laser (488 nm) using Cell Quest software. At the indicated times, two 125 μl aliquots of the cell suspension were transferred to either 125 μl PBS or 125 μl ice-cold PBS containing 5% fatty-acid free BSA (w/v). Cells diluted in buffer only were analyzed immediately (to determine the total cell associated NBD fluorescence) while cells diluted into PBS containing BSA were analyzed after 5 min (representing the cell associated NBD fluorescence protected against BSA back-exchange). Prior to measurement, 1 μl of 1 mg/ml propidium iodide (Molecular Probes, Eugene, OR) was added to the aliquots just before dilution (4-fold in PBS). Ten thousand cells were analyzed without gating during the acquisition. A histogram of the red fluorescence (propidium iodide) was used to exclude dead cells from analysis. Green fluorescence (NBD) of living cells was plotted on a histogram and the mean fluorescence intensity was calculated. The relative amount of each NBD-lipid present in the BSA-treated sample (% protected) was calculated as a percentage of the total amount present in a sample not treated with BSA.

Fluorescence microscopy. Labeled cells were mounted on poly-L-lysine-coated coverslips and examined immediately with an inverse Axiovert 100 standard epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a cooled CCD camera (Coolsnap, visitron systems, Puchheim, Germany) driven by Metamorph software (Universal Imaging, Downingtown, USA). All images were acquired using a Plan-APO 100×/1.3 NA oil objective with the following filter sets: BP 450–490, FT 510, and BP 512–542 (NBD); BP 515–560, FT 580, and LP 590 (FM4-64). To compare the uptake of NBD-lipids with that of the endocytic tracer FM4-64 (Molecular Probes), cells were incubated with 10 μM FM4-64 (from a 50 mM DMSO stock) for 30 min at different temperatures prior to microscopy.

Results

Analysis of NBD-lipid internalization by dithionite

Dithionite reduces the NBD moiety of lipid analogs present in the outer plasma membrane leaflet of labeled cells and destroys their fluorescence. The remaining fluorescence reflects the fraction of analogs internalized into the cells. As shown for NBD-PS, immediately after labeling of cells at 4 °C, dithionite destroyed rapidly all fluorescence (Fig. 1A, curve a), indicative of an exclusive orientation of analogs to the outer leaflet of the plasma membrane. Fluorescence microscopy confirmed that immediately after labeling of cells fluorescence was only associated with the plasma membrane and the flagellum and disappeared completely upon addition of dithionite (Fig. 1B). However, after incubating cells at 28 °C for 20 min prior to analysis, only about 20% of NBD-PS was rapidly accessible to dithionite (Fig. 1A, curve b). Fluorescence microscopy revealed a diffuse distribution of fluorescence in intracellular membranes which was not affected by treatment with dithionite (Fig. 1B). Thus, the dithionite assay allows a rapid determination of the amount of internalized NBD-lipids initially incorporated into the outer leaflet of the parasite plasma membrane.

Glycerophospholipids are rapidly internalized by L. infantum promastigotes

Fig. 2 shows a time course for the internalization of the fluorescent lipid analogs of PC, PE, PS, and SM in promastigotes. When parasites were incubated at 28 °C, the majority of NBD-PC, NBD-PE, and NBD-PS became rapidly protected against dithionite. Within 5 min at 28 °C, about 80% of NBD-PC and 60% of NBD-PS were not accessible to dithionite (Fig. 2A). Redistribution of NBD-PE was very similar to that of NBD-PS (not shown). In contrast, NBD-SM was internalized at a slower rate. After 35 min at 28 °C, only about 40% of NBD-SM was non-accessible.

The difference in uptake between glycerophospholipids and SM was also found when the incubation was performed at 4 °C. Within 30 min about 70% of NBD-PC and 45% of NBD-PS became protected against dithionite quenching, whereas only about 15% of NBD-SM was inaccessible (Fig. 2B). The rate for NBD-PC internalization was faster than that for NBD-PS. Similar results were obtained when BSA was used to deplete the outer leaflet from NBD-lipids and the cell-associated NBD fluorescence was measured by flow cytometry

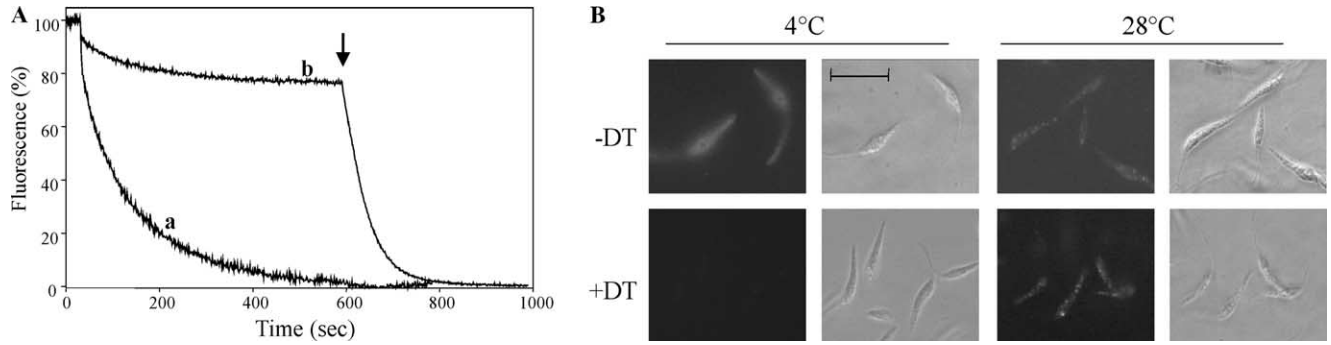

Fig. 1. Accessibility of dithionite to NBD-lipids inserted into the plasma membrane of *Leishmania* promastigotes. (A) Cells were analyzed by fluorescence spectrometry immediately after labeling (a) or after warming to 28 °C for 20 min (b). At $t = 30$ s, dithionite was added to a final concentration of 10 mM and the fluorescence was recorded at 4 °C. To make all label accessible, Triton X-100 was added to a final concentration of 0.5% after 600 s (arrow). (B) Cells were examined by fluorescence microscopy immediately after labeling at 4 °C or after warming to 28 °C for 20 min. Cells were left untreated (−DT) or treated with dithionite at 2 °C (+DT). Bar, 10 μm.

(Figs. 2C and D). This technique also allowed us the exclusion of dead cells. We could not detect any difference in internalization of analogs between promastigotes in the early-log phase and those being in the stationary phase as measured at 28 °C (data not shown).

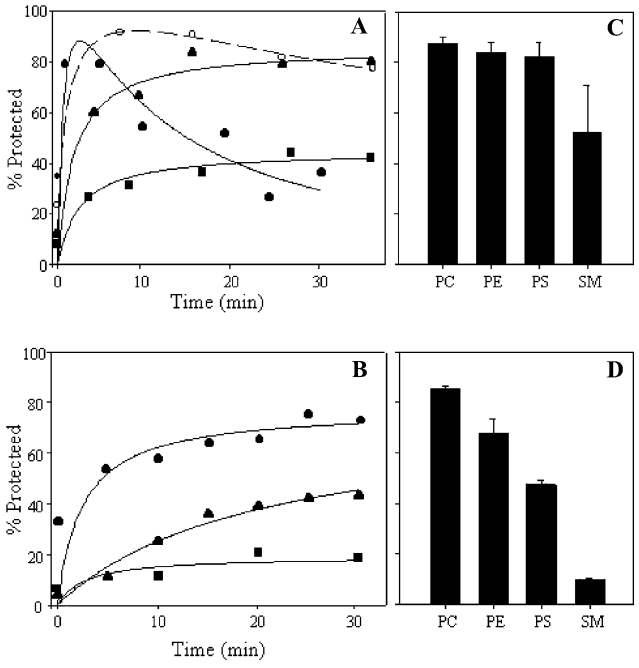

Fig. 2. Internalization of NBD-lipids from the plasma membrane of *Leishmania* promastigotes. Cells were labeled with lipid analogs, washed at 2 °C, and incubated for the indicated times at 28 °C (A,C) or 4 °C (B,D). The fraction of NBD-lipids inside the cells was determined by fluorescence spectrometry using dithionite (A,B) or by flow cytometry using back-exchange to BSA (C,D). Note, at time-zero a small fraction of fluorescent analogs is already inside the cells due to the labeling and washing procedure. In (A,B), the relative fluorescence protected against dithionite is shown for a representative experiment of at least two independent experiments. In (C,D), the relative fluorescence protected against BSA back-exchange is shown; results represent means ± SEM of at least three independent experiments. Filled and open circles—NBD-PC in the absence or presence of DFP, respectively; triangle—NBD-PS; squares—NBD-SM.

Note, data in Fig. 2 refer only to the relative amount of analogs non-accessible to dithionite/BSA, but do not provide information about the total amount of cell associated analogs (see below).

Internalization of NBD-lipids is not caused by metabolic conversion

During the incubation at 28 °C we observed a decrease of the amount of internalized NBD-PC (Fig. 2A). Thin-layer chromatography of lipid extracts revealed that NBD-PC was extensively hydrolyzed into lyso-PC and free NBD-fatty acid, despite the presence of PMSF. The amount of liberated free fluorescent fatty acid was up to 42% after 30 min which accounts for the observed decrease in the amount of internalized analog. In the presence of DFP, the amount of internalized analog reached a maximum of about 90% (Fig. 2A) and hydrolysis of NBD-PC was reduced to 18% after 30 min at 28 °C. When diether NBD-PC, an analog that is not hydrolyzed by phospholipase A2 activities [2], was used, again rapid uptake of the analog was observed reaching a plateau of about 80% (data not shown). For NBD-PS, -PE, and -SM, hydrolysis was less than 8% at 28 °C. At 4 °C, hydrolysis was negligible for all NBD-lipids even 30 min after labeling. Lipid analysis also revealed that the analogs were not subjected to extensive head group modifications. Even at 28 °C, less than 10% of the probes were converted to other NBD-lipids. These results show that the differences in the internalization of the various lipid analogs are not caused by their metabolic conversion at the cell surface.

Internalization of glycerophospholipids requires a translocation step across the plasma membrane

To analyze whether the rapid internalization of PC, PE, and PS occurs by transbilayer movement across the plasma membrane, we examined the intracellular local-

ization of NBD-lipids by fluorescence microscopy after incubation of parasites with analogs for 45 min at 4 °C or 30 min at 28 °C.

When cells were labeled with NBD-SM at 4 °C, only plasma membrane fluorescence was detected which was completely destroyed by treatment with dithionite (Fig. 3A). By contrast, when parasites were labeled with NBD-PC, -PE, and -PS at 4 °C, the majority of fluorescence appeared in internal structures which was not affected after treatment with dithionite (only shown for NBD-PC). Similar differences in the intracellular distribution of NBD-SM and NBD-glycerophospholipids were observed when the cells were back-exchanged with BSA (data not shown). Under the same conditions, the dye FM4-64, an endocytic tracer allowing visualization of membrane traffic from the flagellar pocket to intracellular organelles, was not internalized and remained associated with the plasma membrane showing that NBD-PC, -PE, and -PS are only internalized by transbilayer movement at this temperature.

At 28 °C, endocytic activity of cells became evident by labeling of intracellular compartments with FM 4-64 (Fig. 3B). However, we observed a strong diffuse labeling of the intracellular lumen by NBD-PC, -PE, and -PS. This suggests that internalization of these analogs occurs independently of endocytosis and requires a translocation step across the plasma membrane. We note also labeling of intracellular compartments which may be indicative of an endocytic uptake and/or an enrichment of analogs in organelles subsequent to their translocation across the plasma membrane.

Rapid internalization of glycerophospholipids requires an energy-coupled transporter

After treatment with NEM or depletion of ATP, cells were labeled with NBD-lipids for 30 min at 28 °C (Fig. 4A) or for 45 min at 4 °C (Fig. 4B), washed with BSA to remove analogs from the exoplasmic leaflet of the plasma membrane, and analyzed by flow cytometry. Treatment with NEM (1 mM) caused a 65–85% reduction in the internalization of NBD-PS, -PE, and -PC. A similar reduction in the uptake of these analogs was observed when cells were depleted of ATP to about 5% of control cells. Remarkably, only a modest reduction of uptake was found when ATP was decreased to about 20% of the original level (Fig. 4A, only for 28 °C). In contrast, uptake of NBD-SM was almost negligible in control cells and was only slightly affected at 28 °C by ATP depletion or NEM pretreatment. Pretreatment with NEM slightly enhanced the internalization of NBD-SM but uptake was still lower than that of glycerophospholipids under these conditions, suggesting that NEM enhances the passive movement of the analog across the plasma membrane as described before for erythrocytes and fibroblasts [4].

Based on these findings, we conclude that the uptake of NBD-PC, -PE, and -PS requires an energy-coupled

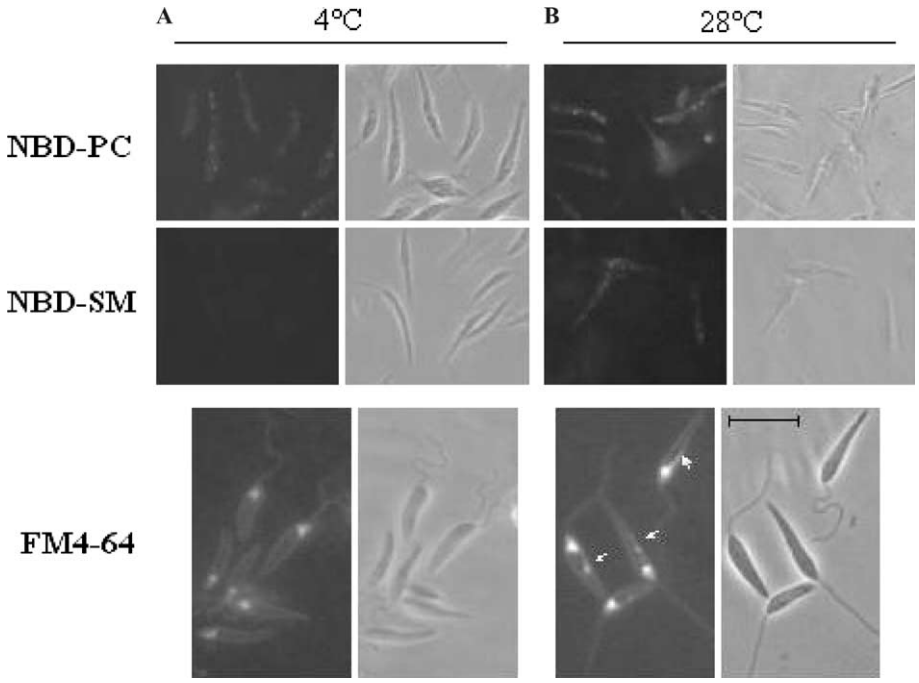

Fig. 3. Fluorescence microscopy of *Leishmania* promastigotes labeled with NBD-lipids. Cells were incubated at 4 °C for 45 min (A) or at 28 °C for 30 min (B) with either NBD-PC, NBD-SM, or FM 4-64 followed by treatment with 10 mM dithionite for 5 min at 4 °C (left—fluorescence images, right—phase contrast). Note, only plasma membrane and flagellar pocket are labeled with FM 4-64 at 4 °C, but no endocytic vesicles are seen as at 28 °C (see arrows). Bar, 8 μm.

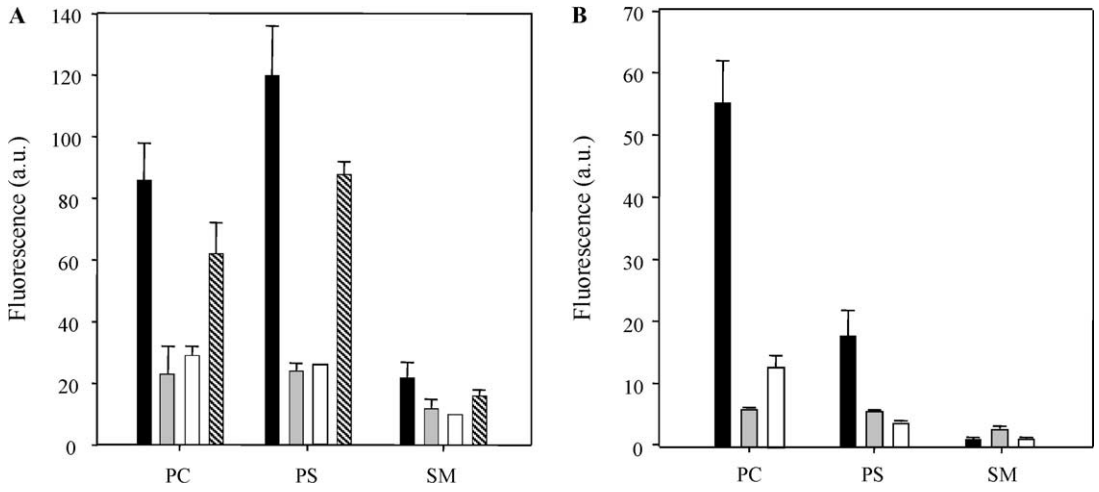

Fig. 4. The effects of NEM pretreatment and ATP depletion on the internalization of NBD-lipids. Cells, pre-incubated in HPMI (black bars), in HPMI containing 1 mM NEM (grey bars) or in glucose-free HPMI containing 20 mM NaN_3 with 5 mM (white bars) and without 2-deoxyglucose (hatched bars) for 30 min at 25 °C, were labeled with NBD-lipids and incubated at 28 °C for 30 min (A) or 4 °C for 45 min (B). Prior to analysis by flow cytometry, cells were diluted 2-fold into PBS with 5% BSA and incubated for 5 min at 4 °C to remove analogs from the exoplasmic leaflet of the plasma membrane. Results represent means ± SEM of at least three independent experiments.

transporter, whereas NBD-SM transfers the plasma membrane via passive diffusion. In order to assess whether lipid analogs, in particular NBD-PC and -PS, are transported by the same protein, we studied the inward movement of NBD-PS in the presence of the non-fluorescent PC analog. To this end, NBD-PC was pretreated with and subsequently separated from dithionite. After 30 min at 28 °C the amount of internalized NBD-PS was reduced by about 70% in the presence of non-fluorescent PC with respect to the control. This indicates that both lipid analogs compete for the same transporter.

Discussion

In this study we found a rapid intracellular uptake of NBD-analogs of PC, PE, and PS in *L. infantum*. The major route of the internalization of these analogs is a protein and energy dependent inward redistribution across the plasma membrane, but not endocytosis, strongly suggesting the presence of a lipid transporter in the plasma membrane of *L. infantum*. In contrast, NBD-SM uptake was low and not coupled to a protein and to energy consumption. We surmise that the SM analog traverses the plasma membrane via passive transbilayer motion.

One may ask whether the fluorescent analogs used are faithful reporters of natural phospholipid movements. There is now convincing evidence to suggest that lipids bearing a fluorescent group are translocated in biological membranes with a selectivity dependent on the head group, and that this reflects a phenomenon which takes place for naturally occurring lipids (for review [6]). In our study, the large difference in the intracellular uptake between the NBD-SM analog with respect to the other lipid analogs demonstrates a selectivity to the head group/glycerol backbone and not to the fluorescent NBD moiety.

Decline of the inward movement of NBD-PS in the presence of non-fluorescent PC analog indicates that the analogs compete for the same lipid transporter. This is supported by the observation that in a *Leishmania donovani* line resistant to the lipid-like drug miltefisone the rapid uptake was impaired for all three analogs (PC, PE, and PS) (Pèrez-Victoria et al., submitted). What is the molecular nature of this transporter? Although beyond our study, we may address this question by comparing the pattern of inward redistribution of lipid analogs in *L. infatum* with those of other eukaryotic cells. As shown for a variety of mammalian cells, e.g. red blood cells, sperm cells, and fibroblasts (for review see [7]), aminophospholipids but not PC and SM are rapidly transported from the exoplasmic to the cytoplasmic leaflet by the ATP-dependent activity of an aminophospholipid translocase (APLT). Although the APLT is not yet known, a candidate for the APLT was cloned from bovine chromaffin granules [8] which was shown to be the first representative of a yet unknown sub-family of P-type ATPases named after the yeast homolog, the Drs2p. We consider it unlikely that a specific transporter such as the mammalian APLT is involved in the rapid uptake of NBD analogs in *L. infantum*, since NBD-PC and NBD-PS compete for the same transporter.

Recently, two novel Drs2p-related P-type ATPases, Dnf1p and Dnf2p, have been identified in the yeast plasma membrane to be involved in the transbilayer movement of PS, PE, and PC [9]. Remarkably, the pattern of phospholipid internalization in yeast cells—

rapid transport of PC, PS, and PE, but slow passive uptake of SM—is very similar to that of *L. infantum*. Interestingly, a closer inspection of the partially sequenced genome of *L. major* (www.ebi.ac.uk/parasites/leish.html) reveals proteins homologous to those of the Drs2p-related P-type ATPase family (F.J. Perez-Victoria and F. Gamarro, unpublished observation). It will be a challenge to elucidate the role of those proteins in lipid transport of *Leishmania*.

In *Leishmania* species, three different classes of ABC transporters are known (for a review see [10]). Two of them are homologous to the mammalian MDR1 Pgp and MRP1, respectively [10,11]. The third group shows high homology with the mammalian ABCA family. Since respective mammalian transporters as ABCB1 (MDR1 Pgp) [12,13], ABCC1 (MRP1) [14], and ABCA1 [15] have been shown to be involved in lipid trafficking, a similar activity might be expected for homologous ABC transporters in parasites. Indeed, a lower accumulation of a fluorescent analog of phosphatidylcholine (BODIPY-C5-PC) and the antiproliferative acting alkyl-lysophospholipids miltefosine and edelfosine in a P-glycoprotein-like transporter overexpressing cell line of *Leishmania tropica* has been observed in comparison to the wild type [16]. Employing fluorescent phospholipid analogs of different head groups (PC, PS, and PE) recent studies have suggested that the ABC transporter LtrABC1.1 related to the human ABCA subfamily is involved in lipid movements across the plasma membrane of the protozoan parasite *L. tropica* (Parodi-Talice et al., submitted). However, since transport activity of ABC transporters is directed towards the exoplasmic leaflet of plasma membranes, we consider it as unlikely that those transporters are responsible for the rapid inward movement of NBD-PC, -PS, and -PE.

Taken together, a challenge of future study will be to identify (i) the transporter(s) mediating a rapid internalization of phospholipids as PC, PS, and PE, across the plasma membrane of parasites, and (ii) their role in infection of cells by parasites. Taking into account the relevance of parasites lipids in invasion of host cells, the lipid transporter(s) can be an essential target of drugs to prevent infection. In the light of current knowledge on lipid transport across plasma membranes of eukaryotes, candidate transporters may belong to the class of P-type ATPases.

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