Plasmodium falciparum-infected erythrocytes utilize a synthetic truncated ceramide precursor for synthesis and secretion of truncated sphingomyelin

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Plasmodium falciparum is an intracellular parasite of human erythrocytes. Parasite development is accompanied by an increase of the phospholipid content of the infected erythrocyte, but it results in a selective decrease of sphingomyelin. We have studied sphingomyelin biosynthesis in infected erythrocytes using as substrate a synthetic radiolabelled ceramide precursor, truncated in both hydrophobic chains. Lysates of infected, unlike those of non-infected, erythrocytes contained sphingomyelin synthase activity, which therefore is of parasite origin. The enzyme activity was associated with a membrane fraction. In contrast to mammalian cells, the parasite did not synthesize detectable levels of glycosphingolipids. In intact infected erythrocytes the ceramide precursor was converted into a correspondingly truncated soluble sphingomyelin which was released into the medium at 37 °C. Release of truncated sphingomyelin was inhibited by low temperature (15 °C) but not by the fungal metabolite brefeldin A which, however, arrests protein export from the parasite. While membranes of mammalian cells, including the plasma membrane of non-infected erythrocytes, are impermeable to truncated sphingomyelin, the membrane of infected erythrocytes allowed passage of the molecule in both directions. The results obtained with the unicellular eukaryote used here as an experimental model are discussed in comparison with sphingomyelin synthesis and transport in mammalian cells.

INTRODUCTION

The malaria parasite *Plasmodium falciparum* is a eukaryotic single cell which invades human erythrocytes (RBCs). The parasite develops within the RBC from a ring stage to a trophozoite and, during schizogony, undergoes multiple nuclear divisions. After the formation of plasma membranes, infectious merozoites are released from the RBC. In the course of parasite development, the phospholipid content of the infected RBC (IRBC) increases 5-fold [1] resulting in an extensive network of tubovesicular membranes (TVM) within the IRBC cytoplasm [2,3]. Furthermore, the phospholipid composition of the erythrocyte membrane (RBCM) is altered fundamentally [1,4–7], which may be responsible for an increased permeability of the RBCM. An increased uptake of glucose [8], nucleosides [9], amino acids [10,11] and certain anions [12,13] into IRBC has been demonstrated. In contrast to the parasite, the host cell is unable to synthesize proteins or lipids *de novo*. The morphological and physiological changes observed within the IRBC and at the RBCM, respectively, coincide with the secretion of proteins and lipids from the parasite cell [14–18]. An understanding of the contribution of parasite-derived molecules to the biochemical and morphological alterations induced in the host cell requires a detailed analysis of the biosynthetic pathways of molecules which are exported from the parasite into the host RBC.

Sphingolipids are major constituents of the plasma membrane and the membranes of secretory organelles in higher eukaryotes. They are synthesized from ceramide precursors which are either modified to sphingomyelin or, alternatively, to glycosphingolipids. In mammalian cells, sphingomyelin synthase activity has been localized to the *cis*-Golgi compartment [19,20]. In IRBCs, the sphingomyelin content of the RBCM is reduced by 47% as compared with non-infected RBCs [1], and very little sphingomyelin is present in membranes of the parasite [4]. Nevertheless, labelling experiments using *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]aminohexanoylsphingosine (C6-NBD-Cer) showed conversion of this molecule into *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]aminohexanoylsphingosine-1-phosphocholine (C6-NBD-Sm) in IRBCs by an enzymic activity that is not present in non-infected RBCs, thus providing evidence for sphingomyelin synthase activity in IRBCs [21]. Microscopical analyses revealed trapping of C6-NBD-Sm in a perinuclear location inside the parasite cell as well as in the TVM, suggesting sphingomyelin synthase activity in two distinct locations within the IRBC [22].

An alternative substrate for the study of sphingolipid biosynthesis in mammalian cells has been a radiolabelled truncated ceramide derivative ([^{3}H]tCer) in which both hydrophobic chains were reduced to 8 C-atoms. [^{3}H]tCer is converted into radiolabelled truncated sphingomyelin ([^{3}H]tSPH) or to the corresponding glycosphingolipid [23]. While [^{3}H]tCer freely passes through cellular membranes, [^{3}H]tSPH, owing to its polar headgroup, is trapped in the lumen of the endomembrane system [23]. Owing to the truncation of both hydrophobic tails, [^{3}H]tCer and [^{3}H]tSPH are soluble molecules with physical properties that differ significantly from those of C6-NBD-Sm in which only a single fatty acid chain is truncated and which therefore remains membrane associated [24]. C6-NBD-Sm is transported to the plasma membrane and internalized into endocytic vesicles, whereas [^{3}H]tSPH accumulates in the medium when exocytotic vesicles fuse with the plasma membrane. The solubility of [^{3}H]tSPH allows quantification of both synthesis and release of the molecule from intact cells. In the present report we have used [^{3}H]tCer as substrate to study sphingolipid synthesis in IRBCs. On the one hand our results confirm previous reports on

Abbreviations used: BFA, brefeldin A; C6-NBD-Cer, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]aminohexanoylsphingosine; C6-NBD-Sm, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]aminohexanoylsphingosine-1-phosphocholine; IRBC, infected erythrocyte; RBC, erythrocyte; RBCM, erythrocyte membrane; [^{3}H]tCer, radiolabelled truncated ceramide; [^{3}H]tGlcCer, radiolabelled truncated glucosylceramide; [^{3}H]tSPH, radiolabelled truncated sphingomyelin; TVM, network of tubovesicular membranes.

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sphingomyelin synthase activity in IRBCs. In addition we find that in contrast to C6-NBD-Sm, [^{3}H]tSPH is released from intact IRBCs.

MATERIALS AND METHODS

Materials

UDP-glucose was purchased from Boehringer–Mannheim. Brefeldin A (BFA) was obtained from Sigma and prepared as a 1 mg/ml stock solution in ethanol. [^{3}H]tCer (50 μCi/nmol) and unlabelled truncated ceramide were synthesized according to [23]. Radiolabelled truncated glucosylceramide ([^{3}H]tGlcCer) and [^{3}H]tSPH were isolated from the medium of Chinese hamster ovary (CHO) cells labelled with [^{3}H]tCer and characterized as described in [23]. L-[^{35}S]Methionine was obtained from Amersham.

Parasite culture

P. falciparum, strain FCBR, was grown in human RBCs, blood group A$^+$, in RPMI medium containing 10% (v/v) human serum. Parasites were maintained at synchronous developmental stages by weekly passage through plasmagel [25] and sorbitol lysis [26].

Incubation of lysates of IRBCs with [^{3}H]tCer and analysis of products

IRBCs were enriched to a parasitaemia of 60–80% [25] and washed twice in PBS. IRBCs and, as a control, non-infected RBCs (10^7 cells respectively) were lysed by three cycles of freezing and thawing and adjusted with PBS to a volume of 9 μl, routinely at a protein concentration of 140 mg/ml. Golgi membranes were prepared from rat liver [27] and diluted in 9 μl of PBS to a protein concentration of 0.5 mg/ml. The samples were incubated with [^{3}H]tCer and products analysed essentially as described in [27]. Briefly, 2 nmol of unlabelled tCer and 2 μCi of [^{3}H]tCer were dried in a gentle stream of nitrogen and redissolved in 10 μl of 0.6 M NaCl, 0.1 M EDTA. One μl of the mixture was added to 9 μl of the various samples and incubated at 37 °C for 1 h under shaking. To determine synthesis of [^{3}H]tGlcCer, UDP-glucose was added to a final concentration of 4 mM. After 1 h the reaction was stopped by the addition of 10 μl of isopropanol, and the mixture was kept on ice for 5 min. The solution was centrifuged at 15800 *g* for 5 min at 4 °C. The supernatant (5 μl aliquot) was analysed by TLC on Whatman silica-gel plates LK6 in butan-2-one/acetone/water/formic acid (30:3:5:0.04, by vol.). The chromatograms were analysed using an automatic TLC-2D-analyser (Berthold, Wildbad, Germany). Signals were determined for 1 h (c.p.h.) which correlate to 0.5% of signal yield detectable by liquid scintillation counting [20,27].

Fractionation of IRBCs

Lysis of IRBCs with saponin allows the separation of the host-cell cytoplasm from a fraction containing intact parasites [28,29]. IRBCs (6×10^7) were collected by centrifugation and incubated on ice in 1.5 vol. of 0.15% ice-cold saponin (Serva) in RPMI. After 5 min, the sample was centrifuged at 1300 *g* for 5 min at 4 °C. The supernatant was collected. The pellet was lysed by three cycles of freezing and thawing, extracted with several volumes of high-salt buffer (50 mM Hepes, pH 7.5/0.5 M KCl/5 mM dithiothreitol/50 mM lysine/3 mM $MgCl_2$), and membranes were sedimented at 4 °C by centrifugation at 15800 *g* for 30 min. To determine the presence of sphingomyelin synthase activity in the individual fractions, aliquots from each fraction, each corresponding to 5×10^6 IRBCs, were tested for their ability to convert [^{3}H]tCer into [^{3}H]tSPH as described above. Serum-free medium from a culture of 1×10^7 IRBCs was evaporated, resuspended in 10 μl of 50% isopropanol and assayed for sphingomyelin synthase activity, likewise.

Labelling of intact IRBCs with [^{3}H]tCer

Parasites were maintained synchronous by regular sorbitol lysis and passage through plasmagel. At 26 h after invasion, IRBCs were enriched by the plasmagel procedure [25]. Parasitaemia and the developmental stages of the parasites were determined microscopically. IRBCs, at a parasitaemia of 60–80% (consisting of less than 3% polynucleated schizonts and more than 95% trophozoite-infected erythrocytes), were washed twice in RPMI and resuspended in RPMI at a concentration of 2×10^8 IRBC/ml. Resuspended IRBCs were adjusted to a temperature of 37 °C or 15 °C. A mixture of [^{3}H]tCer (0.45 μCi) and unlabelled truncated ceramide (0.75 nmol) was dried under nitrogen, dissolved in 1.5 μl of 0.6 M NaCl/0.1 M EDTA and added to samples containing 1.5×10^7 IRBCs for each time point. IRBCs were cultured at 37 °C or at 15 °C. At each time point 10^7 IRBCs and a corresponding volume of medium were analysed for the distribution of [^{3}H]tSPH in the cellular fraction and in the medium. The remainder, containing 5×10^6 labelled IRBC in a corresponding volume of medium, was analysed for the distribution of haemoglobin. For the analysis of [^{3}H]tSPH the cell pellet was lysed in 50% isopropanol; the medium (50 μl) was mixed with an equal volume of isopropanol. Samples were left on ice for at least 5 min, the liquid was evaporated and the remainder dissolved in 10 μl of 50% isopropanol. Aliquots (5 μl) of each sample were analysed by TLC as described above. For the analysis of haemoglobin, 5×10^6 IRBCs were separated from a corresponding volume of culture medium and resuspended in 25 μl of RPMI. Medium and resuspended IRBCs were each mixed with 975 μl of water and the haemoglobin concentration in the respective samples was determined spectrophotometrically at 412 nm. For treatment with BFA, IRBCs were preincubated with 5 μg/ml BFA for 15 min at 37 °C prior to the addition of a mixture of [^{3}H]tCer and unlabelled truncated ceramide. At several time points, IRBCs were harvested and cells and medium were processed as described above.

Pulse–chase labelling of IRBCs

IRBCs (7×10^8) were incubated in 3.5 ml of RPMI at 15 °C, in the presence of [^{3}H]tCer. After 1 h, IRBCs were chilled on ice and washed twice in RPMI to remove [^{3}H]tCer from the medium. Subsequently, cells were incubated at 15 °C for 30 min to allow conversion of residual [^{3}H]tCer. IRBCs were washed twice and resuspended in an equal volume of RPMI, followed by incubation at 37 °C. After several time points the distribution of [^{3}H]tSPH in the medium and in the cells was determined.

Assay for the permeability of non-infected RBCs and IRBCs for exogenously added [^{3}H]tSPH

Intact IRBCs and intact non-infected RBCs (10^8 cells each) were incubated with 0.04 μCi of [^{3}H]tSPH at various temperatures (4 °C, 15 °C, 37 °C). Cells were incubated in equal volumes of serum-free medium and harvested after several time points. The suspension was chilled on ice, centrifuged at 1300 *g* for 0.5 min and cells were washed twice with 50 μl of ice-cold RPMI. The medium and the washing solutions were pooled. Cells and

medium, respectively, were mixed with 1 vol. of isopropanol and assayed for [^{3}H]tSPH as described above.

Immunoprecipitation of metabolically labelled protein

Labelling of IRBCs with L-[^{35}S]methionine and immunoprecipitation of the parasite glycophorin-binding protein after saponin lysis of IRBCs were carried out as described in detail elsewhere [29].

RESULTS

[^{3}H]tSPH, but not [^{3}H]tGlcCer, is synthesized by lysates from IRBCs

Microscopical and chromatographic analyses of intact IRBCs incubated with C6-NBD-Cer have revealed the presence of sphingomyelin synthase activity in IRBCs, which was located predominantly in the parasite cell. Some enzyme activity was also found in the intra-erythrocytic TVM [22]. In mammalian cells, [^{3}H]tCer is converted into [^{3}H]tSPH and to [^{3}H]tGlcCer, as well as to higher glycosphingolipids. Glucosyl ceramide synthase is located at the cytosolic face of the Golgi [27], and it catalyses the synthesis of [^{3}H]tGlcCer from [^{3}H]tCer and UDP-glucose, a reaction parallel to the synthesis of [^{3}H]tSPH. To investigate whether [^{3}H]tCer is utilized by parasite sphingomyelin synthase, lysates from non-infected RBCs, IRBCs and a rat liver Golgi fraction were compared for their ability to convert [^{3}H]tCer into [^{3}H]tSPH and [^{3}H]tGlcCer, respectively. In the absence of lysate, [^{3}H]tCer was detected as a fast-migrating spot of radioactivity on TLC plates (Figure 1a, lane 1). It was not converted in a lysate of RBCs (Figure 1a, lane 2). In a lysate of IRBC and in the Golgi fraction, [^{3}H]tCer was converted into a hydrophilic product which was retained close to the starting point (Figure 1a, lanes 3 and 4), and which has been identified previously as [^{3}H]tSPH [23]. In addition to [^{3}H]tSPH, [^{3}H]tGlcCer was synthesized by the Golgi fraction, but not by the IRBC lysate. To rule out the possibility that IRBCs contain an inhibitor of glucosyl ceramide synthase, synthesis of [^{3}H]tGlcCer was determined in a mixture containing equal parts of IRBC and Golgi lysates (Figure 1a, lane 5). The presence of the IRBC lysate did not inhibit synthesis of [^{3}H]tGlcCer. Likewise, upon addition of UDP-galactose, lysates from IRBCs did not produce detectable amounts of [^{3}H]tGalCer (data not shown).

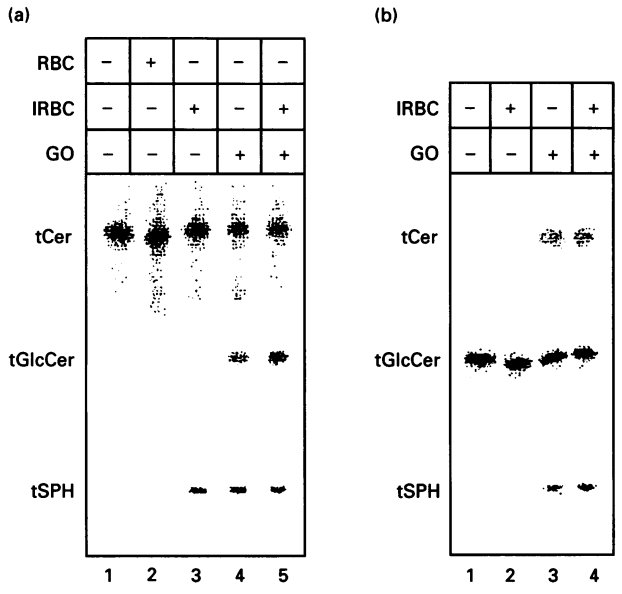

Figure 1 Cell-free synthesis of [^{3}H]tSPH

(**a**) Chromatographic analysis of lysates from non-infected erythrocytes (RBCs), infected erythrocytes (IRBCs), a Golgi fraction from rat liver (GO) and a mixture of IRBCs and GO. The lysates were incubated for 1 h with [^{3}H]tCer, in the presence of UDP-glucose. The reaction mixtures were separated by TLC and analysed by an automatic TLC-2D-analyser. (**b**) Chromatographic analysis of lysates incubated with [^{3}H]tGlcCer. Lysates of IRBCs, GO, and a mixture containing equal volumes of IRBCs and GO, were incubated for 1 h with [^{3}H]tGlcCer, separated by TLC and radioactive products were analysed as described for (**a**).

In mammalian cells, [^{3}H]tGlcCer can be hydrolysed to [^{3}H]tCer. We tested the formal possibility that in *P. falciparum* the activity of a hydrolase exceeds that of glucosyl ceramide synthase, thus resulting in undetectable levels of [^{3}H]tGlcCer. Purified [^{3}H]tGlcCer, at a final concentration of 20 μM, was added to a lysate of IRBCs, to a fraction of rat liver Golgi and to a mixture consisting of equal parts of intact Golgi membranes and lysed IRBCs. [^{3}H]tGlcCer was not converted in the lysate of IRBCs (Figure 1b, lanes 1 and 2), suggesting that a hydrolase activity was not present in the lysate. In contrast, in the Golgi fraction and in the mixture of Golgi and IRBC lysate, [^{3}H]tGlcCer was partially hydrolysed to [^{3}H]tCer, a part of which was subsequently converted into [^{3}H]tSPH. In conclusion, IRBCs contain sphingomyelin synthase activity which is not present in non-infected RBCs; however, in contrast to mammalian cells, IRBCs do not have detectable levels of glucosyl ceramide synthase.

Sphingomyelin synthase activity is membrane associated in mammalian cells [19,20]. In order to investigate membrane association of the parasite enzyme, IRBCs were treated with saponin, a procedure which results in a preferential lysis of the membrane of IRBCs, allowing the separation of mostly intact parasites from the soluble contents of the IRBC cytoplasm [28,29]. Subsequently, a membrane fraction of the parasites was obtained by freezing and thawing of the parasite cells, followed by extraction of the membranes with a buffer containing 0.5 M KCl [30]. This procedure allows efficient separation of soluble proteins contained in the erythrocyte cytoplasm from soluble proteins of the parasite cell and the isolation of a membrane fraction. The fractions used for the following experiments were routinely assayed for the distribution of specific marker proteins as described previously [31]. The conversion of [^{3}H]tCer into [^{3}H]tSPH was compared in a total lysate of IRBCs, a sample of the host-cell cytosol, a lysate of parasite cells released from IRBCs and the fraction of parasite membranes (Table 1). No enzyme activity was detectable in the medium of IRBCs (data not shown). The IRBC cytosol contained $> 70\%$ of the total protein, but only 11% of the synthase activity found in a total lysate of IRBCs was detected in this fraction. In agreement with previous studies, using C6-NBD-Cer as a substrate [22], most of the activity was associated with the fraction containing intact parasites. This activity was quantitatively recovered in the membrane fraction obtained after lysis of the released parasites which corresponds to an enrichment of enzymic activity of more than 60-fold. Extraction of the membranes with 0.1 M Na_2CO_3, at pH 11.5, resulted in a complete loss of enzymic activity, both in the membrane fraction and in the fraction of proteins solubilized at high pH (data not shown).

[^{3}H]tSPH is released from intact IRBCs into the culture medium

In mammalian cells, [^{3}H]tSPH is transported via the secretory pathway and therefore secreted into the culture medium. We investigated whether [^{3}H]tSPH synthesized by the intracellular

Table 1 Distribution of sphingomyelin synthase activity in infected erythrocytes

To obtain a total lysate of IRBCs, cells were lysed by freezing and thawing. For subfractionation, IRBCs were lysed with saponin and centrifuged. The supernatant (IRBC cytosol) was collected and the fraction containing released parasites was lysed by freezing and thawing. For preparation of a membrane fraction, membranes of released and lysed parasites were extracted with a high-salt buffer and collected by centrifugation. The protein content, corresponding to an equivalent of 5×10^6 IRBCs, was determined in the total lysate and in the individual fractions. The synthesis of [^{3}H]tSPH was determined in the total lysate and in the individual fractions, each sample corresponding to 5×10^6 IRBCs.

	Protein (μg)	Total protein (%)	[^{3}H]tSPH (c.p.h.)	Synthesis of [^{3}H]tSPH (%)
IRBC	148	100	4522	100
IRBC cytosol	108	73	480	11
Released parasites	27	18	2684	59
Membrane fraction	< 1	< 1	2707	60

parasite was released from intact IRBCs. IRBCs were incubated in the presence of [^{3}H]tCer, and the amount of [^{3}H]tSPH in intact IRBCs and in the medium was monitored over a period of 2.5 h (Figure 2a). The release of haemoglobin into the medium is the most sensitive parameter with which to assess intactness of IRBCs. The amount of haemoglobin released from 5×10^6 labelled IRBCs into the incubation medium was determined at each time point. Consistently, less than 1 % of the haemoglobin was released into the medium. [^{3}H]tSPH inside IRBCs reached a steady-state level after 20 min. In the medium, the amount of [^{3}H]tSPH increased continuously. Thus, [^{3}H]tSPH was translocated beyond the RBCM. In order to determine the influence of temperature on the release of [^{3}H]tSPH, experiments were carried out in parallel at 15 °C. At the lower temperature [^{3}H]tSPH accumulated inside IRBCs, and only small amounts were released into the medium (Figure 2b). The kinetics of [^{3}H]tSPH synthesis were similar at 37 °C and 15 °C, indicating that neither entry of [^{3}H]tCer into IRBCs nor sphingomyelin synthase activity were reduced at the lowered temperature. Figure 2(c) illustrates, on the basis of three independent experiments, the ratio of [^{3}H]tSPH in the medium and in the cellular fraction at 15 °C and at 37 °C. The amounts of [^{3}H]tSPH in the medium of IRBCs incubated at 37 °C were consistently 4–6-fold higher than in the medium of cells incubated at 15 °C.

BFA does not affect synthesis or release of [^{3}H]tSPH

A potent inhibitor of protein export in mammalian cells and in *P. falciparum* is the fungal metabolite BFA [32]. In mammalian cells, the rate of sphingolipid synthesis was enhanced by BFA [33–35]. In some experimental systems transport of sphingomyelin to the cell surface was inhibited by BFA [33,35], whereas in a different report [34] BFA had no effect on the transport of sphingomyelin. To investigate the effects of BFA on the secretion of [^{3}H]tSPH from IRBCs, cells were cultured in the presence of BFA and [^{3}H]tCer. The distribution of [^{3}H]tSPH in the medium and in IRBCs was analysed after several time intervals (Figure 3a). In comparison with untreated control cells, BFA had no significant effect on the synthesis of [^{3}H]tSPH nor its release from IRBCs. In order to assess the previously described effect of BFA on protein secretion from the parasite, the distribution of the glycophorin-binding protein, a marker for a parasite-encoded protein which is secreted into the IRBC cytosol, was investigated. Proteins of IRBCs were metabolically labelled in the presence or in the absence of BFA, and cells were lysed with saponin. The distribution of the glycophorin-binding protein in intact parasites and in the IRBC cytosol, respectively, was analysed by immunoprecipitation using a specific antiserum [29]. In the presence of

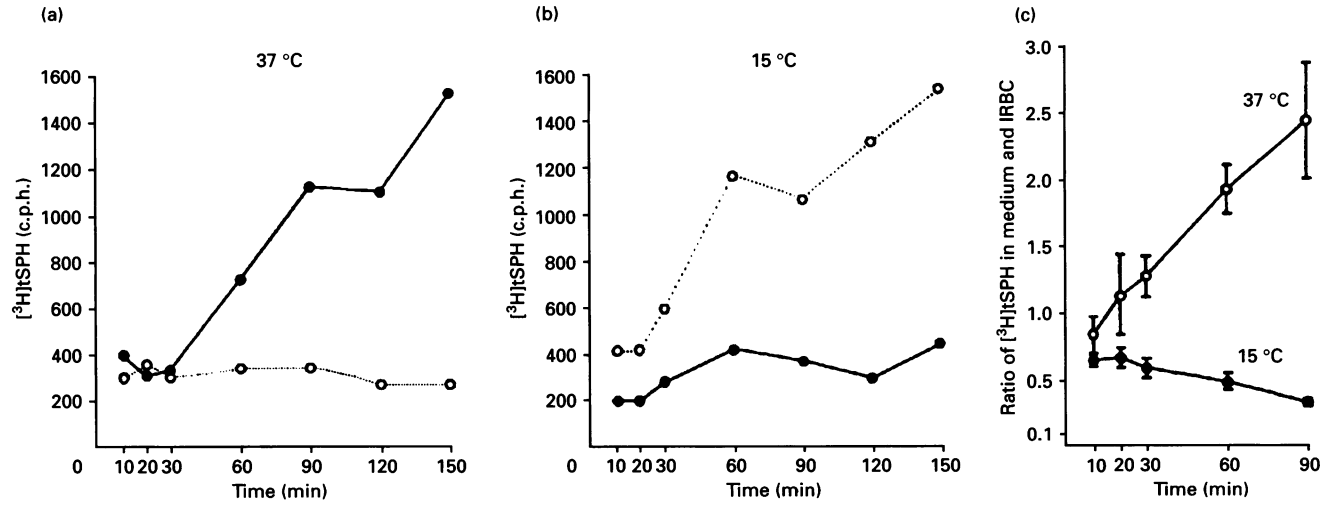

Figure 2 [^{3}H]tSPH is released from intact IRBCs

IRBCs (2×10^8 cells per ml of RPMI) were incubated with [^{3}H]tCer, either at 37 °C or at 15 °C. At several time points, IRBCs were harvested and a corresponding volume of medium was collected. Counts incorporated into [^{3}H]tSPH were determined for 1 h in the cellular fraction and in the medium sampled at each time point. (**a**) Distribution of [^{3}H]tSPH in IRBCs and medium at 37 °C. ○, [^{3}H]tSPH in the cellular fraction; ●, [^{3}H]tSPH in the medium. (**b**) Distribution of [^{3}H]tSPH in IRBCs and medium at 15 °C: ○, [^{3}H]tSPH in the cellular fraction; ●, [^{3}H]tSPH in the medium. (**c**) IRBCs were incubated with [^{3}H]tCer at 37 °C or at 15 °C. Cells and medium were collected at various time points and [^{3}H]tSPH was determined as described above. The ratio of [^{3}H]tSPH in medium to cells at each time point was calculated. On the vertical axis, the statistical means of data obtained from three parallel experiments are plotted. The vertical bars indicate the standard deviation. ●, ratios determined at 15 °C; ○, ratios determined at 37 °C.

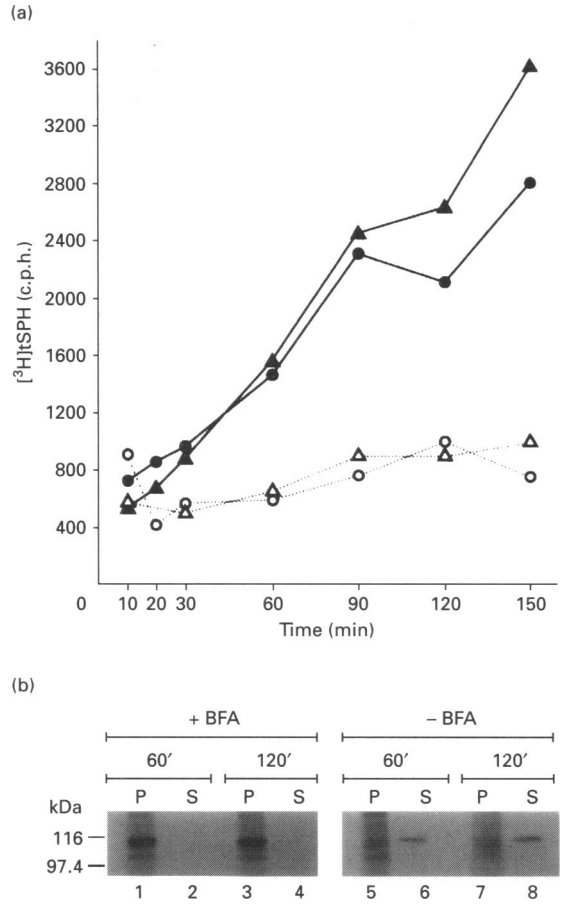

Figure 3 Synthesis of [^{3}H]tSPH and release into the culture medium are not affected by BFA

(**a**) IRBCs were labelled with [^{3}H]tCer as described for Figure 2. In one series of experiments BFA (5 μg/ml) was added to the medium. The distribution of [^{3}H]tSPH in cells and medium was determined in a time-course experiment as described for Figure 2. ●, [^{3}H]tSPH in the medium of control cells; ○, [^{3}H]tSPH in cells; ▲, [^{3}H]tSPH in the medium of cells treated with BFA; △, [^{3}H]tSPH in cells treated with BFA. (**b**) Effect of BFA on protein export from the parasite cell. Proteins synthesized by IRBCs were metabolically labelled, either in the presence or in the absence of BFA. IRBCs were lysed by saponin treatment and separated into a fraction containing intact parasites (P) and into a fraction containing host-cell cytosol (S). From each fraction, proteins were immunoprecipitated using an antiserum specific to the parasite glycophorin-binding protein [29]. Precipitated proteins were separated on an SDS/10%-polyacrylamide gel and visualized by autoradiography. The numbers on the left indicate the respective molecular-mass markers in kDa.

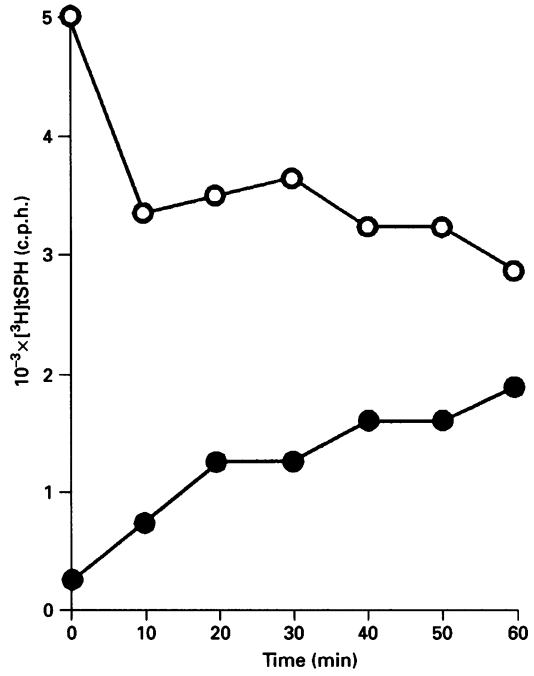

Figure 4 Pulse–chase experiment

IRBCs were incubated with [^{3}H]tCer at 15 °C to accumulate intracellular [^{3}H]tSPH. Cells were washed in ice-cold RPMI, and incubated in an equal volume of medium containing no [^{3}H]tCer. The loss of [^{3}H]tSPH from IRBCs and its appearance in the medium was monitored in a time-course experiment. ○, [^{3}H]tSPH in the cellular fraction; ●, [^{3}H]tSPH in the medium.

BFA, the protein was retained within the parasite (Figure 3b, lanes 1–4). In the absence of BFA, the protein was recovered from the IRBC cytosol (Figure 3b, lanes 5–8).

Kinetics of [^{3}H]tSPH release from intact IRBCs

In a pulse–chase experiment the rate of [^{3}H]tSPH release from IRBCs was determined. IRBCs were cultured in the presence of [^{3}H]tCer at 15 °C to minimize release of [^{3}H]tSPH. Cells were transferred to medium without [^{3}H]tCer and incubated to allow conversion of [^{3}H]tCer into [^{3}H]tSPH. Subsequently, IRBCs were mixed with an equal volume of medium, warmed to 37 °C and [^{3}H]tSPH concentrations were determined in the medium and in IRBCs after several time points (Figure 4). At the onset of the chase, [^{3}H]tSPH was almost quantitatively recovered in IRBCs, and within 60 min approximately 40 % of total [^{3}H]tSPH was recovered from the medium. Most of [^{3}H]tSPH was released within the first 20 min of the chase after which the rate of release slowed down considerably, in accordance with an equilibration of the intracellular and extracellular [^{3}H]tSPH pools. Indistinguishable results were obtained when, during the chase period, ATP was depleted in the presence of 50 mM 2-deoxy-D-glucose and 3 mM sodium azide.

The RBCM becomes permeable to [^{3}H]tSPH by the infection

In mammalian cells, the membranes of the secretory compartments, including the plasma membrane, are impermeable barriers for [^{3}H]tSPH [23]. We tested whether the same principle applies to the plasma membranes of non-infected RBCs and IRBCs. RBCs and IRBCs were each incubated in medium containing purified [^{3}H]tSPH, at a ratio of one volume of medium to one volume of cells. The distribution of [^{3}H]tSPH in the medium and in the cellular fraction was determined over a period of 90 min (Figure 5). After incubation of non-infected RBCs all detectable [^{3}H]tSPH was recovered in the medium, demonstrating that the membrane of RBCs is impermeable to [^{3}H]tSPH. When IRBCs were incubated with [^{3}H]tSPH, increasing amounts of [^{3}H]tSPH were detectable in the cellular fraction in a time-course experiment. To exclude the formal possibility that [^{3}H]tSPH adhered to the outer face of the IRBC membrane, IRBCs were incubated with [^{3}H]tSPH at 4 °C and at 15 °C, temperatures which should reduce the fluidity of the membrane without affecting adherence. The amount of [^{3}H]tSPH recovered with the cellular fraction was significantly lower at 15 °C than at 37 °C. At 4 °C no [^{3}H]tSPH was detected in the cellular fraction.

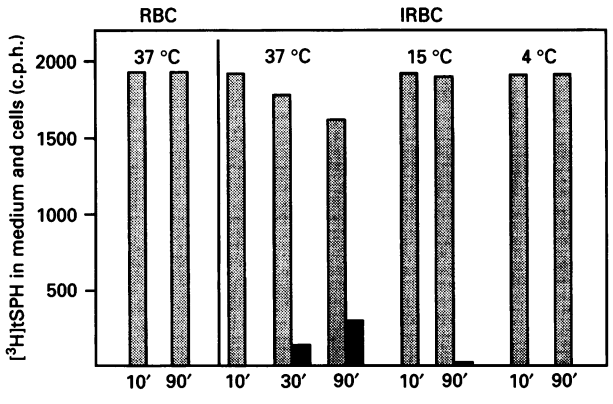

Figure 5 The membrane of IRBCs is permeable for [^{3}H]tSPH

Non-infected RBCs were incubated at 37 °C, IRBCs were incubated at 37 °C, 15 °C and 4 °C in RPMI (ratio of medium:cells = 1:1) containing [^{3}H]tSPH. After various time points, cells and medium were collected, and the distribution of [^{3}H]tSPH was determined. The stippled bars represent radioactivity in the medium; the closed bars indicate radioactivity recovered in the cellular fraction. The left-hand scale indicates counts incorporated into [^{3}H]tSPH.

These observations indicate an increase in the permeability of the membrane of IRBCs to [^{3}H]tSPH, most likely as a result of the parasite infection. In order to provide comparable culture conditions for RBCs and IRBCs, RBCs, prior to the incubation with [^{3}H]tSPH, were maintained for several hours in medium withdrawn from IRBCs. The medium obtained from IRBCs did not cause a permeability of RBCs for [^{3}H]tSPH.

DISCUSSION

P. falciparum induces major biochemical and physiological alterations of its host cell that can be seen as a result of the parasite's biosynthetic activities. While the total phospholipid content of IRBCs increases 5-fold as compared with non-infected RBCs, some phospholipids, namely sphingomyelin, appear to be selectively diminished [1]. Reports on the conversion of C6-NBD-Cer into C6-NBD-Sm [21] prompted us to study sphingomyelin synthesis using [^{3}H]tCer as a radiolabelled, soluble substrate which facilitates sequestration of soluble [^{3}H]tSPH in a fraction that is distinct from the site of its synthesis, thus allowing quantification of sphingolipid production. Lysates of IRBCs, in contrast to lysates of non-infected RBCs, contain sphingomyelin synthase activity which, like that of mammalian cells, is membrane associated. In contrast to most mammalian cells, the parasite lysate did not synthesize detectable levels of glucosyl ceramide and contains no activity that would hydrolyse the glycosphingolipid. When synthesized by intact IRBCs, [^{3}H]tSPH was released into the medium via a pathway which was not sensitive to BFA but which could be blocked by incubation of the cells at low temperature.

Sphingomyelin synthase activity in IRBCs has been described previously, but no translocation of C6-NBD-Sm into the RBCM has been observed [21]. However, some controversy exists in the literature as to whether other phospholipids originating from the intracellular parasite are translocated across the RBCM [16,36]. The appearance of [^{3}H]tSPH in the medium of IRBCs was unexpected because biological membranes are generally believed to be impermeable to [^{3}H]tSPH and no vesicular transport pathway from the IRBC cytoplasm, reminiscent of the mechanism of [^{3}H]tSPH secretion from mammalian cells, has been established experimentally. Since IRBCs, in contrast to non-infected RBCs, are permeable to exogenously added [^{3}H]tSPH, we attribute the release of [^{3}H]tSPH to an increased permeability of the RBCM. Several previous reports have described an increase in the permeability of the membrane of IRBCs for a wide range of molecules [1,9-11,13]. It is unknown whether the enhanced transfer of naturally occurring molecules such as glucose, nucleosides and amino acids (for which a specific transport across the RBCM under normal physiological conditions can be envisaged) is mediated by parasite-derived proteins or by transporter proteins of the host. In this context it is noteworthy that [^{3}H]tSPH is a synthetic molecule which may become a suitable marker to study novel biochemical properties of the RBCM.

In mammalian cells, sphingomyelin biosynthesis almost exclusively occurs in the Golgi apparatus [19,20] and, consequently, transport of sphingomyelin to the cell surface is inhibited by BFA, at least in some experimental systems [33,35]. In IRBCs, morphological analyses have demonstrated sphingomyelin synthase activity in two distinct locations; (i) a perinuclear site inside the parasite and (ii) the TVM [22]. Although BFA inhibits protein export from the parasite and leads to a redistribution of the parasite homologue of *ERD2* [37] the drug has no effect on the intracellular distribution of sphingomyelin synthase activity, at least at the light microscopical level [37]. Likewise, our observation that export of [^{3}H]tSPH is not impaired by BFA suggests that synthesis of [^{3}H]tSPH occurs in a compartment of the parasite's secretory pathway which is insensitive to treatment with BFA. Since a morphologically distinct Golgi apparatus, as characterized by stacked cisternae, has not been identified; and since Golgi-specific post-translational modifications are not found in proteins of erythrocytic-stage parasites, it is currently not possible to relate the intracellular location of parasite sphingomyelin synthase to that of the mammalian enzyme. However, it is intriguing to speculate that sphingomyelin biosynthesis in organisms which are evolutionary distant from higher eukaryotes and which have no requirement for a functionally complex Golgi apparatus, resides at a site of the secretory pathway that follows the BFA-sensitive compartment. The decrease in sphingomyelin content in IRBCs, despite a high activity of parasite sphingomyelin synthase, suggests metabolic pathways which require large amounts of sphingomyelin and which have yet to be identified.

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