Lipid metabolism in *Trypanosoma brucei*: utilization of myristate and myristoyllysophosphatidylcholine for myristoylation of glycosyl phosphatidylinositols

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Myristate is the exclusive fatty acid species in the glycosyl phosphatidylinositol (GPI) anchor of the *Trypanosoma brucei* variant surface glycoprotein (VSG). [^3H]Myristate can be incorporated into *T. brucei* GPIs by two distinct processes known as fatty acid remodelling and myristate exchange. Myristoyllysophosphatidylcholine (M-LPC) can also serve as a myristate donor for VSG in trypanosomes [Bowes, Samad, Jiang, Weaver and Mellors (1993) J. Biol. Chem. **268**, 13885–13892]. We have studied in detail the myristoylation of GPIs using a [^3H]M-LPC substrate. Labelling of VSG and free GPIs by [^3H]M-LPC in cultured trypanosomes occurred at the same rate as with [^3H]myristate. Concurrent with GPI labelling, there was rapid hydrolysis of [^3H]M-LPC to generate extracellular [^3H]myristate.

Experiments in a trypanosomal cell-free system indicated that GPI labelling by fatty acid remodelling and myristate exchange was also equally efficient with [^3H]M-LPC and [^3H]myristate. Furthermore, both ATP and CoA are required for the myristoylation of GPIs by [^3H]M-LPC. These experiments suggest that GPI myristoylation from M-LPC involves hydrolysis of M-LPC to free myristate. To address the physiological importance of myristate and M-LPC in VSG myristoylation, we radiolabelled trypanosomes *in vivo* with both substrates in medium containing serum, and found that [^3H]myristate labelled VSG and GPIs more efficiently. Thus, VSG myristoylation by free myristate may be favoured in bloodstream trypanosome infections.

INTRODUCTION

Myristic acid is crucial to *Trypanosoma brucei*, the parasite responsible for African sleeping sickness in humans and nagana in cattle. This fatty acid is a component of the trypanosome variant surface glycoprotein (VSG), an abundant molecule which forms the parasite's surface coat. The trypanosome evades eradication by the host's immune response through antigenic variation, a process in which the expression of one VSG is switched to that of another (see [1] for review).

VSG is tethered to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor. The core structure of this anchor, ethanolamine-*P*-Man₃-GlcN-phosphatidylinositol [2], is highly conserved in eukaryotes. The VSG GPI anchor is unique, however, in that its lipid component is solely dimyristoylglycerol [3]. Trypanosomes have evolved two pathways to ensure myristoylation of the VSG GPI. The GPI precursor is initially synthesized in a non-myristoylated form, known as glycolipid A'. Through fatty acid remodelling reactions involving sequential deacylation and reacylation, glycolipid A' becomes myristoylated, forming the GPI precursor glycolipid A [4]. This GPI species is then linked to the C-terminus of the newly synthesized VSG. A second pathway for GPI anchor myristoylation, termed myristate exchange, operates predominantly on VSG protein [5,6]. This pathway, involving removal of the myristoyl groups from the GPI anchor and their replacement by other molecules of myristate, apparently uses enzymic machinery distinct from that of remodelling and occurs in a different subcellular com-

partment. Myristate exchange may be a proofreading or repair system to ensure complete myristoylation of VSG.

Although the reasons for myristate specificity in VSG remain unknown, trypanosomes have an enormous requirement for this fatty acid since each cell bears 10^7 VSG molecules [7,8]. Its importance to the parasite is highlighted by the selective toxicity of myristic acid analogues to *T. brucei* in culture [9,10]. Despite the critical role myristate plays in trypanosome metabolism, *de novo* synthesis of myristate or any other fatty acid does not occur in bloodstream forms of *T. brucei* [11–13]. Therefore all fatty acids, including myristate, must be imported from the host's plasma. Myristate is relatively scarce in mammalian plasma; its concentration as a non-esterified fatty acid (NEFA) is about $1.6\ \mu\text{M}$ in rat serum [14] and around $6\ \mu\text{M}$ in human plasma, and it generally comprises about 1 % of the total fatty acids present in the lipid species of human plasma (A. Moser, personal communication). To grow to a density of 10^9 parasites/ml (a cell concentration which is easily attained in the blood of infected laboratory rats) and fully myristoylate its 10^7 VSG molecules, the trypanosome must use more myristate than is normally available in the bloodstream as NEFA. Therefore, the task of scavenging myristate by the trypanosome appears formidable.

Bowes et al. [15] have raised the possibility that free myristate may not be the only significant source of myristate for trypanosomes. They showed that 1-acyl-2-hydroxy-*sn*-glycero-3-phosphorylcholine (lysophosphatidylcholine or LPC) is a good source of fatty acids for *T. brucei*. The authors propose that the trypanosome hydrolyses a portion of exogenous LPC (by a

Abbreviations used: VSG, variant surface glycoprotein; GPI, glycosyl phosphatidylinositol; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; M-LPC, myristoyllysophosphatidylcholine; GPC, L- α -glycerophosphorylcholine; MeOH, methanol; NEFA, non-esterified fatty acid.

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plasma membrane phospholipase) to produce a cell-associated NEFA. The acyl-CoA derivative of this NEFA is then formed, which can be used to synthesize phosphatidylcholine (PC) through the acylation of endogenous or exogenous LPC. Of special interest was the finding that myristate from 1-[^{14}C]-myristoyllysophosphatidylcholine (M-LPC) was incorporated into the VSG GPI anchor. LPC is present in human plasma at concentrations between 100 and 250 μM [16], and fatty acid composition analysis of human plasma has shown that M-LPC constitutes 4.4% of the total plasma LPC in normal subjects [17]. Therefore, myristate and M-LPC should be present at comparable concentrations in plasma. Due to the vital role played by myristate in trypanosome metabolism, it is essential to examine the importance of exogenous M-LPC to *T. brucei*.

We have studied the mechanism of GPI myristoylation by M-LPC and compared the utilization of this substrate with that of myristate. Our findings indicate that GPI myristoylation by M-LPC first involves hydrolysis to generate extracellular myristate. This myristate is then imported and converted into its CoA derivative, which serves as a donor for GPI myristoylation. Our results also suggest that free myristate may be kinetically preferred to M-LPC as a myristate donor for trypanosomal GPIs during an infection by bloodform trypanosomes.

MATERIALS AND METHODS

Chemicals

Where not otherwise specified, materials were from Sigma. Organic solvents were from J. T. Baker or Aldrich, and 1,1'-carbonyldiimidazole and 1,8-diazabicyclo[5.4.0]undec-7-ene were from Aldrich. Non-radioactive lipids and lipid precursors were from Avanti Polar Lipids, Inc. 1,2-Di[^{14}C]myristoyl-*sn*-glycero-3-phosphorylcholine (116 mCi/mmol) was from DuPont-New England Nuclear, and [9,10- ^3H]myristate (51–54 Ci/mmol) was from Amersham Corporation.

Synthesis of 1-[9,10- ^3H]myristoyl-2-hydroxy-*sn*-glycero-3-phosphorylcholine ([^3H]M-LPC)

This compound was synthesized by the method of Hermetter and Paltauf [18] with several modifications. [9,10- ^3H]Myristate (2.2 mCi, 43.1 nmol) and 1,1'-carbonyldiimidazole (1 mg, 6.2 μmol) were added to a 5 ml glass vial, along with one or two 5 Å molecular sieves. The vial was stoppered, placed under a nitrogen atmosphere, and CH_2Cl_2 (0.2 ml) was added via syringe. This reaction mixture was shaken for 45 min at room temperature, generating myristoyl imidazolide. In a separate vial were added L- α -glycerophosphorylcholine (GPC, 2.8 mg, 11 μmol), 1,8-diazabicyclo[5.4.0]undec-7-ene (7.1 mg, 47 μmol), a few molecular sieves, and DMSO (0.4 ml, previously dried over molecular sieves). The GPC solution was then directly transferred via syringe to the vial containing myristoyl imidazolide, and the reaction mixture was shaken for 2.5 days at room temperature. At this molar ratio of GPC to myristate (256:1), monoacylation is favoured. Water (1 ml) was then added to the reaction mixture, which was extracted once with 1.2 ml and twice with 0.6 ml of $\text{CHCl}_3/\text{MeOH}$ (2:1, v/v). The combined organic phases were concentrated first under a stream of N_2 and then by Speedvac. [^3H]M-LPC was isolated by preparative TLC developed with $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (10:10:3, by vol). Authentic [^{14}C]M-LPC, formed by bee-venom phospholipase A_2 digestion of 1,2-di[^{14}C]myristoyl-*sn*-glycero-3-phosphorylcholine, was used as a marker. Radioactivity was detected with a Bioscan System 2000 imaging scanner. The product (which comigrated with the [^{14}C]M-LPC standard) was scraped from the plate and then

extracted from silica with $\text{MeOH}/\text{pyridine}/\text{H}_2\text{O}$ (2:1:1, by vol.). [^3H]M-LPC (339 μCi) was obtained; yield 15.4% based on [^3H]myristate. Under these conditions, >95% of the GPC-coupled radiolabelled material is recovered as M-LPC (results not shown). NMR studies have shown that LPC exists as a mixture of 1-acyl (90%) and 2-acyl (10%) LPC at equilibrium [19].

Metabolic labelling of trypanosomes with ^3H -labelled lipids

For *in vivo* labellings, the radiolabelled lipid was dried under nitrogen, dissolved in 95% EtOH, and then diluted with serum-free RPMI 1640 medium (Gibco) containing 0.5 mg/ml defatted BSA (the final percentage of EtOH did not exceed 1%). *T. brucei* parasites (MITat 1.2, variant 221, from G. A. M. Cross, Rockefeller University, New York, N.Y., U.S.A.) were maintained in long-term culture at 37 °C in HMI-9 medium [20] containing 10% fetal calf serum (Gibco BRL) and 10% Serum Plus (JHR Biosciences, Lenexa, KS, U.S.A.). Late log-phase parasites were washed once in ice-cold bicine-buffered saline (50 mM bicine, pH 8.0/50 mM NaCl/5 mM KCl/55 mM glucose) and resuspended (10^7 parasites/ml) in the radiolabelling medium described above. After incubation at 37 °C, samples were placed on ice for 5 min, centrifuged at 1200 *g* for 10 min at 4 °C, and washed once with 0.5–1 ml of bicine-buffered saline.

Labelling in the trypanosome cell-free system

Membranes from cloned ILTat 1.3 trypanosomes were prepared as in [21] with the modifications described in [5]. Labelling with [^3H]myristate and [^3H]M-LPC was performed as previously described [5].

Lipid and VSG analysis

Lipid analysis was performed essentially as described previously [4,22]. Briefly, cells or membranes were extracted with $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (10:10:3, by vol.) and centrifuged at 11000 *g* for 10 min at 4 °C. The organic supernatant was dried under nitrogen and partitioned between water-saturated butan-1-ol and water. The butanol extraction was then repeated, the organic layers were dried under nitrogen, lipids were redissolved in $\text{CHCl}_3/\text{MeOH}$ (1:1, v/v), and the samples were analysed on pre-dried Silica Gel 60 TLC plates (E. M. Science) developed with $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (10:10:3, by vol.). VSG analysis was conducted on the $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ -insoluble pellet from trypanosome cell extracts, which was dissolved in 1% SDS by heating at 37 °C for 30 min, before scintillation counting or electrophoresis. For analysis of the radiolabel remaining in the medium after *in vivo* labelling, aliquots of the post-labelling medium (obtained by centrifugation of labelled trypanosomes at 1200 *g* for 10 min at 4 °C) were either directly analysed by scintillation counting or extracted twice with water-saturated butan-1-ol; butanol extracts were dried under a stream of nitrogen and the lipid was fractionated by TLC as above. When [^3H]myristate or [^3H]M-LPC labellings were performed in the cell-free system, dried 10:10:3 extracts were boiled for 8 min in 50 μl of 25 mM MnCl_2 to hydrolyse [^3H]myristoyl-CoA (which can obscure GPIs on TLC plates) before butan-1-ol/water partitioning [5]. The TLC plates were assayed for radioactivity by scanning and then fluorographed. The scanning efficiency of each radiolabelled lipid species is different due to differential quenching; to convert scanner units into actual concentrations, samples in control experiments were scraped from the TLC plate and assayed by scintillation counting as described previously [5].

RESULTS

Incorporation of [^3H]myristate into VSG from ^3H -labelled NEFA and LPC

Bowes et al. showed previously that [^{14}C]myristate from [^{14}C]M-LPC metabolically labels trypanosome VSG [15]. We have investigated the kinetics of VSG myristoylation by [^3H]M-LPC and [^3H]myristate (both at 78 nM) by labelling trypanosomes in serum-free medium and using SDS/PAGE and scintillation counting to analyse VSG. Figure 1 shows that the myristoylation of VSG by these two substrates is virtually identical.

Labelling of lipids *in vivo* with [^3H]myristate and [^3H]M-LPC

We next compared the labelling of free GPIs by [^3H]myristate and [^3H]M-LPC *in vivo*. After labelling, we extracted the lipids and analysed them by TLC. A fluorograph of typical TLCs (after a 5 min labelling) is shown in Figure 2(A) (labelling with [^3H]myristate, lane 1, and with [^3H]M-LPC, lane 2). Also shown are the kinetics of labelling of trypanosomal lipid species by [^3H]myristate (Figure 2B) and by [^3H]M-LPC (Figure 2C); panels A, B and C are from the same experiment. We found that labelling of the GPIs glycolipid A (the VSG precursor) and glycolipid C (a GPI identical with glycolipid A, except that it possesses a fatty acylated inositol [23]) are strikingly similar, whether [^3H]M-LPC or [^3H]myristate was the precursor. The major difference between the two experiments was the labelling of PC (other lipids were labelled similarly). With [^3H]M-LPC, the kinetics of PC labelling were biphasic (Figure 2C); PC labelling by [^3H]myristate showed an approximately linear increase with time (Figure 2B).

Figure 1 *In vivo* radiolabelling of VSG with [^3H]M-LPC and [^3H]myristate

T. brucei parasites (5×10^6 parasites/sample, 10^7 cells/ml) were labelled as described in the Materials and methods section with [^3H]M-LPC or [^3H]myristate (both at $4 \mu\text{Ci/ml}$, 78 nM). [^3H]Myristate-labelled VSG was isolated and samples were prepared for analysis as described in the Materials and methods section; half of the sample was fractionated by 10% SDS/PAGE, while the other half was quantified by scintillation counting. The inset shows the part of the gel where VSG runs (VSG accounts for greater than 90% of the radiolabelled protein in these experiments). The gel was treated with En^3Hance (Du Pont), dried, and fluorographed using preflashed Kodak X-Omat film. The graph shows the data obtained from scintillation counting. Open circles indicate labelling by [^3H]M-LPC, while closed circles represent labelling by [^3H]myristate. The radioactivity measured at each time point was multiplied by the appropriate factor to reflect pmol VSG/ 5×10^6 cells. In a control experiment we demonstrated that less than 1% of the [^3H]M-LPC was hydrolysed in the culture medium in the absence of trypanosomes at 37 °C for 60 min.

Trypanosomes quickly metabolize [^3H]M-LPC during the course of the experiment. For example, no trace of this compound remained in trypanosome lipids in the 5 min labelling shown in Figure 2(A) (lane 2, M-LPC runs just below glycolipid C on TLC developed with this solvent system). Most of the radioactivity in the cell is incorporated into PC, with smaller amounts converted into NEFAs, other lipids and VSG. To study the metabolism of [^3H]M-LPC in more detail, we used TLC to analyse the radioactivity remaining in the culture medium and compared it with cell-associated radioactivity (Figure 2D). Parasites incubated with [^3H]M-LPC show a rapid decrease in [^3H]M-LPC remaining in the medium, with virtually all being consumed in 10 min. However, most of this radioactivity does not immediately become cell-associated. Concomitant with the disappearance of [^3H]M-LPC is a burst in the production of extracellular free myristate (the identity of the fatty acid was confirmed by radiochemical analysis of its methyl ester by reverse-phase TLC ([14], results not shown)). This result is in contrast to a previous hypothesis, which suggested that the NEFA produced by LPC hydrolysis was intracellular [15]. Control experiments showed that the hydrolytic activity is cell-associated and is not secreted into the medium. Therefore, this NEFA is most likely produced by phospholipase cleavage of [^3H]M-LPC at the trypanosomal plasma membrane [15,24]. Trypanosomes labelled initially with [^3H]myristate consume this fatty acid at a fairly constant rate, with the radioactivity removed from the medium appearing in cellular lipids (results not shown).

Labelling of GPIs with [^3H]myristate and [^3H]M-LPC in a cell-free system

Our laboratory has developed a cell-free system containing washed trypanosome membranes, which permits GPI myristoylation by either fatty acid remodelling [4] or myristate exchange [5]. The substrate for both reactions is myristoyl-CoA. Our next objective was to determine whether [^3H]M-LPC could also serve as a substrate for GPI myristoylation in the cell-free system. Unexpectedly, we found that M-LPC was as effective as myristate in labelling GPIs by either the remodelling or exchange reactions.

As shown previously [4], incubation of the cell-free system with [^3H]myristate (in the presence of ATP, CoA, UDP-GlcNAc and GDP-Man) resulted in labelling of glycolipid A' (a partially remodelled GPI containing a single myristate that is made only from products of *de novo* GPI biosynthesis) and also of glycolipids A and C (Figure 3, lane 1). The latter two GPIs could have been labelled either by remodelling or exchange. Incubation with [^3H]myristate, ATP, and CoA, in the absence of UDP-GlcNAc and GDP-Man, resulted in labelling (by exchange only [5]) of glycolipids A and C (lane 3). Similar experiments with [^3H]M-LPC (in the presence of UDP-GlcNAc, GDP-Man, ATP and CoA) (lane 2) and in the absence of sugar nucleotides (lane 4), indicated that labelling of GPIs was even more efficient than with [^3H]myristate; in some other experiments, labelling by both substrates was at about the same level (for example, in Figure 4, compare lanes 1 and 5).

Because higher concentrations of radiolabel were used in this experiment compared with Figure 2, substantial amounts of [^3H]M-LPC remained in samples labelled with this lipid. We therefore could not assess the labelling of glycolipid C in the experiment shown as it was obscured by the [^3H]M-LPC substrate. In another experiment (results not shown), these species were separated using $\text{CHCl}_3/\text{MeOH}/\text{conc. NH}_4\text{OH}$ (10:10:3, by vol.) as a solvent system (R_f of M-LPC, 0.34; R_f of glycolipid C, 0.17). Levels of glycolipid C labelled by [^3H]M-LPC were

Figure 2 *In vivo* radiolabelling of trypanosome lipids with [^3H]M-LPC and [^3H]myristate

Trypanosomes (5×10^6 cell equivalents/sample, 10^7 cell equivalents/ml) were labelled with [^3H]M-LPC or [^3H]myristate (both at $4 \mu\text{Ci/ml}$, 78 nM), processed as described in the Materials and methods section, and the radiolabelled lipids were fractionated by TLC. (A) Fluorogram of TLC plate showing trypanosomal lipids labelled after a 5 min incubation. Lane 1, [^3H]myristate labelling; lane 2, [^3H]M-LPC labelling. Glycolipid A (A) is the GPI anchor precursor; glycolipid C (C) is a related GPI possessing an acylated inositol (the identities of these species was verified by digestion with GPI-specific phospholipase C [36] and GPI-specific phospholipase D [37]). O, origin; F, front; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FFA, non-esterified fatty acid; NL, neutral lipids. PC labels as a doublet due to heterogeneous fatty acyl group composition [15], as seen in lane 1. A PC doublet is not observed in lane 2 because the approx. 7-fold greater labelling of PC by M-LPC at this time point precludes resolution of the two PC species. Since lanes 1 and 2 were not adjacent to one another on the original TLC plate, the upper band of the PC doublet in lane 1 ran a bit higher than the PC species in lane 2. (B, C) Kinetics of labelling lipids by [^3H]myristate (B) and [^3H]M-LPC (C). Trypanosomes were labelled with [^3H]M-LPC or [^3H]myristate and lipid extracts were fractionated by TLC as described in (A). Lipid species were identified and quantified with a Bioscan 2000 Imaging Scanner. (A–C) show data from the same experiment. (D) Analysis of radiolabel remaining in the medium after labelling with [^3H]M-LPC. Live trypanosomes were labelled with [^3H]M-LPC under conditions identical with those described for (A–C). Aliquots of the medium were analysed for radioactivity as described in the Materials and methods section. Total medium radioactivity was determined by scintillation counting, the ratio of myristate to M-LPC in the medium was determined by TLC scanning of fractionated lipids and confirmed by autoradiography, and total cell associated radioactivity was measured after dissolving the cell pellet in $100 \mu\text{l}$ of 1% (v/v) SDS. The recovery of the radiolabel was 98–100% at each time point. At higher concentrations of M-LPC ($\geq 220 \text{ nM}$), incomplete hydrolysis was observed after 45 min at 37°C (results not shown).

Figure 3 Labelling of GPI intermediates in the cell-free system by [^3H]myristate and [^3H]M-LPC

Trypanosome membranes were prepared and labelled under conditions described previously [4,22]. The cell-free system (2×10^7 cell equivalents/lane) was incubated (20 min, 37°C) in assay buffer containing Hepes (50 mM, pH 7.4), KCl (25 mM), MgCl_2 (5 mM), MnCl_2 (5 mM), dithiothreitol (0.5 mM), ATP (1.0 mM) and CoA (0.2 mM) in the presence (lanes 1 and 2) or the absence (lanes 3 and 4) of 1 mM GDP-mannose and 1 mM UDP-GlcNAc with $50 \mu\text{Ci/ml}$ of either [^3H]myristate (lanes 1 and 3) or [^3H]M-LPC (lanes 2 and 4). Membranes were extracted, lipids were fractionated on TLC plates, and the plate was fluorographed [4]. The bands above and below PC (marked by asterisks) were formed when radiolabelled dried lipid samples from $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ extractions of the trypanosomal lysate were boiled in aqueous solutions containing MnCl_2 (in order to destroy acyl-CoA, which obscures GPI species). These species were also formed when pure acyl-CoA was boiled under the same conditions. Abbreviations are as in Figure 2(A) except for A'', which is a partially remodelled GPI containing one myristate.

similar to those labelled by [^3H]myristate, paralleling the results with glycolipids A and A''.

As expected, incubation with [^3H]M-LPC also resulted in extensive labelling of PC (Figure 3, lanes 2 and 4). [^3H]M-LPC was also hydrolysed to free [^3H]myristate, although the concentration of free [^3H]myristate (lanes 2 and 4) was about 4-fold less (as determined by TLC scanning) than that observed when the lysate was incubated with [^3H]myristate (lanes 1 and 3). The kinetics of labelling of GPI species in the cell lysate increases in a linear fashion over a 20 min time course when the lysate is labelled with either [^3H]M-LPC or [^3H]myristate (results not shown). In contrast, PC is labelled very rapidly by M-LPC but not by myristate; synthesis of this species is complete within 1 min after the addition of [^3H]M-LPC to the lysate (results not shown). Myristoyl-CoA is also produced from both [^3H]myristate and [^3H]M-LPC. This species runs as a smear in the glycolipid A and A'' region of the TLC plate and obscures these GPIs, so samples are boiled in an aqueous solution of MnCl_2 to hydrolyse the acyl-CoA. This boiling procedure also results in the production of the species indicated by asterisks in Figure 3. Unboiled samples from an experiment, performed under conditions identical with those employed in Figure 3, gave equivalent levels of myristoyl-CoA from [^3H]myristate and [^3H]M-LPC; the [^3H]myristoyl-CoA measured in this experiment was roughly 10-fold greater than the amount of [^3H]glycolipids A and A'' combined (results not shown).

Figure 4 ATP and CoA requirement for GPI biosynthesis from myristate and M-LPC

Labelling of the cell-free system was performed as described in Figure 3 in the presence of GDP-mannose and UDP-GlcNAc with [^3H]myristate (lanes 1–4) or [^3H]M-LPC (lanes 5–8). Lanes 1 and 5, both 1.0 mM ATP and 0.2 mM CoA present; lanes 2 and 6, 0.2 mM CoA present, no ATP; lanes 3 and 7, 1.0 mM ATP present, no CoA; lanes 4 and 8, no ATP or CoA. The species marked by asterisks (*) were formed from [^3H]acyl-CoA upon boiling, as described in the legend to Figure 3.

Figure 5 *In vivo* VSG labelling by [^3H]myristate or [^3H]M-LPC in the presence of serum

VSG isolation and fractionation by SDS/PAGE was performed as described in Figure 1 after live trypanosomes (5×10^6 parasites/sample, 10^7 cells/ml) were labelled for 30 min in RPMI 1640 medium containing 0.5 mg/ml fatty acid-free BSA and fetal calf serum in the presence of 195 nM ($10 \mu\text{Ci/ml}$) [^3H]myristate (lanes 1–4) or [^3H]M-LPC (lanes 5–8). Lanes 1 and 5, no serum; lanes 2 and 6, 10% (v/v) serum; lanes 3 and 7, 25% (v/v) serum; lanes 4 and 8, 50% (v/v) serum. Incubation in fetal calf serum containing no trypanosomes at 37 °C for 60 min resulted in hydrolysis of less than 5% of the [^3H]M-LPC substrate.

ATP and CoA are required for GPI myristoylation in the cell-free system using a [^3H]M-LPC donor

We next investigated the cofactor requirement for GPI biosynthesis with M-LPC in the cell-free system (Figure 4). Using [^3H]myristate as a substrate (lanes 1–4), appreciable labelling of lipid species is evident only in the presence of both ATP and CoA (lane 1) and does not occur with CoA alone (lane 2), ATP alone (lane 3), or without CoA or ATP (lane 4). When [^3H]M-LPC is used (lanes 5–8), the presence of both ATP and CoA is also required for glycolipid A biosynthesis (lane 5), and there is no appreciable GPI labelling in the presence of CoA alone (lane 6), ATP alone (lane 7), or in the absence of both cofactors (lane 8). PC labelling in the absence of ATP and CoA (lane 8) is decreased 5-fold relative to the labelling of this species in the presence of these cofactors (lane 5). The presence of small amounts of

[^3H]PC labelled by [^3H]M-LPC in the absence of ATP and CoA (lane 8) may be due to a low level of fatty acyl-CoA remaining in the washed trypanosome membranes.

Effect of added serum on *in vivo* labelling of VSG

Our *in vivo* experiments performed so far, conducted on trypanosomes cultured in the absence of serum, have shown that [^3H]myristate and [^3H]M-LPC are equally efficient in labelling VSG and free GPIs (Figures 1 and 2). Similarly, labelling of GPIs in the cell-free system occurs efficiently with both substrates (Figure 3). To assess myristate and M-LPC utilization under more physiological conditions, we examined the effect of added serum on the labelling of VSG by these lipids *in vivo* (Figure 5). We labelled trypanosomes with [^3H]myristate (lanes 1–4) and [^3H]M-LPC (lanes 5–8) in medium containing 0% (lanes 1 and 5), 10% (lanes 2 and 6), 25% (lanes 3 and 7) and 50% (lanes 4 and 8) serum. Although labelling of VSG by the two substrates is equally efficient when no serum is present, added serum decreases VSG labelling by [^3H]M-LPC to a greater extent than such labelling by [^3H]myristate. When the labelling medium contained 25% serum, VSG labelling by [^3H]myristate was inhibited $64 \pm 9\%$ ($n = 3$), whereas labelling by [^3H]M-LPC was decreased $92 \pm 2\%$ ($n = 3$) (results expressed as the mean $\pm$ S.D.; the experiments used to calculate inhibition of VSG labelling at 25% serum included the one shown in Figure 5). Serum had similar effects on labelling of the free GPIs glycolipids A and C (results not shown).

To examine the basis of the preference for myristate over M-LPC in VSG myristoylation observed in Figure 5, we have performed competition experiments. Trypanosomes were labelled with 360 nM [^3H]myristate in RPMI 1640 medium containing 0.5 mg/ml BSA in the presence of non-radioactive myristate or non-radioactive M-LPC (both at 2.5 μM). We found that myristate was a much more efficient inhibitor of VSG myristoylation. Added non-radioactive myristate inhibited VSG myristoylation by $76 \pm 1\%$ ($n = 2$), while M-LPC resulted in only a $30 \pm 6\%$ ($n = 2$) inhibition.

DISCUSSION

The African trypanosome has a huge requirement for myristic acid, needed for acylation of the GPI anchor of its VSG. Although free myristate is a component of mammalian plasma and has been found to efficiently label VSG and trypanosome GPIs [3,4], Bowes et al. demonstrated that LPC is also an efficient fatty acid donor for trypanosomes in culture [15]. Furthermore, they found that addition of [^{14}C]M-LPC to a trypanosome culture results in labelling of VSG [15]. Since the concentration of M-LPC in plasma is comparable to that of free myristate (see the Introduction), M-LPC could be a significant source of myristate for trypanosome GPI biosynthesis. Because of the critical role of myristate for the trypanosome, it was essential that we investigate both the relative efficiencies of myristate and M-LPC in supporting GPI myristoylation and the mechanism of GPI myristoylation by M-LPC.

In experiments with live trypanosomes labelled in a serum-free medium, we found that low concentrations of [^3H]M-LPC labelled VSG and free GPIs at a rate virtually identical with that of [^3H]myristate. A major difference in lipid labelling from the two donors was that radioactivity was incorporated into PC much more rapidly from [^3H]M-LPC than from [^3H]myristate. We also observed the rapid hydrolysis of [^3H]M-LPC to generate extracellular myristate, which was itself eventually taken up by the trypanosome and incorporated into GPIs and other lipids

(Figures 1 and 2). LPC hydrolysis by trypanosomes was observed previously, but the NEFA produced was suggested to be intracellular [15]. This conclusion was reached perhaps because the lipid-isolation procedure used by the previous authors differed from ours. In the earlier report, a butanol extraction was performed immediately after live trypanosomes were incubated with radiolabelled LPC [15], whereas we first centrifuged labelled cells to separate them from the labelling medium and washed the cells before lipid extraction. Complete hydrolysis of [^3H]M-LPC was observed in Figure 2(D) even though 10 % of the radiolabel is likely to be the 2-acyl isomer. In the time required for complete hydrolysis of M-LPC in this experiment (10 min), very little spontaneous acyl migration would occur [19]. We were unable to determine the positional specificity of the trypanosomal lyso-phospholipase activity using radiochemical amounts of M-LPC, which would be insufficient for analysis by NMR spectroscopy. However, van den Bosch et al. showed that both 1-acyl and 2-acyl LPC were susceptible to hydrolysis by a rat liver lyso-phospholipase activity [25], and the same could be true in *T. brucei*. It is interesting to note that the rapid phase of PC labelling and, as a result, the rapid phase of incorporation of radiolabel from [^3H]M-LPC into the trypanosome cell, corresponded to the time in which large amounts of [^3H]M-LPC were present in the radiolabelling medium (Figures 2C and 2D). Not unexpectedly, when [^3H]myristate was the only radiolabel remaining in the culture medium after [^3H]M-LPC hydrolysis, consumption and cellular incorporation of radioactivity into PC were strikingly similar to that observed when [^3H]myristate was the added substrate (Figure 2B).

Surprisingly, [^3H]M-LPC also labelled GPIs in the cell-free system, by either remodelling or myristate exchange, as efficiently as did [^3H]myristate (Figure 3). This comparable labelling occurred even though the concentration of [^3H]myristate produced from [^3H]M-LPC (as determined by TLC scanning) was one-quarter of that present when [^3H]myristate was added directly (compare Figure 3, lanes 1 and 2 or 3 and 4). In the cell-free system, the apparent K_m for myristoyl-CoA in both the remodelling and exchange reactions was very low, around 10 nM [5]. The concentration of radiolabel in this experiment was nearly 100-fold higher than these K_m values. Most importantly, the levels of [^3H]myristoyl-CoA produced from [^3H]myristate and [^3H]M-LPC were equivalent in this experiment and were much greater than the levels of myristoylated GPIs (results not shown). Therefore the free myristoyl-CoA concentration was likely to be saturating, whether it was formed from free [^3H]myristate or [^3H]M-LPC.

There appear to be three general mechanistic possibilities for the myristoylation of GPIs by M-LPC. In the simplest case, which we believe to be correct, M-LPC is first hydrolysed to free myristate (as demonstrated in Figure 2D) and then the free myristate is converted into myristoyl-CoA in a reaction dependent upon added ATP and CoA (the requirement for both of these cofactors was demonstrated in Figure 4). We have previously shown that exogenous [^3H]myristoyl-CoA supports GPI myristoylation efficiently either by remodelling [4] or exchange [5]. Alternative possible mechanisms are that M-LPC itself, or PC, which is rapidly formed from M-LPC (Figures 2 and 3 and [15]), could be a direct myristoyl donor. There are well documented examples of CoA-independent and CoA-dependent transacylases (reviewed in [26]), and the inositol acylation of mammalian GPIs is known to be a CoA-dependent (but ATP-independent) process, presumably involving transfer of the acyl group from a phospholipid to the GPI [27]. A reaction similar to the latter may occur in trypanosomes [28]. It is unlikely that M-LPC is a direct donor of myristate in our case because Figure 4 shows that both ATP

Figure 6 Model for M-LPC utilization and GPI myristoylation in African trypanosomes

and CoA are required for GPI myristoylation from [^3H]M-LPC. There is also no indication that PC is a direct donor. Figure 4 (lane 8) shows that although some PC was labelled in the absence of ATP and CoA, no radioactivity was transferred to GPIs under these conditions. This is not surprising, as chemical analysis of trypanosome lipids revealed that less than 1 % of the acyl residues of PCs are myristoyl [14]. Given the data presented here, and that [^3H]myristoyl-CoA is a known substrate for GPI myristoylation [4], we believe GPI myristoylation by M-LPC involves a myristoyl-CoA intermediate.

Free myristate is channelled efficiently into GPI myristoylation reactions in the presence of serum, as we have shown previously [14]. M-LPC, although still available for GPI myristoylation, was disfavoured as such a donor when serum (which contains NEFA and LPC bound to albumin, as well as lipoproteins) was present (Figure 5). The factors influencing fatty acid uptake by cells are complex and are not yet clearly understood [29–33], and it is beyond the scope of the present study to examine the effect of such factors on NEFA and LPC uptake in the trypanosome. Nonetheless, the possibility that M-LPC could become sequestered by binding to serum proteins and thus be less available for uptake by the trypanosome compared with myristate seems unlikely because of the data obtained from our competition experiment presented at the end of the Results section. This experiment, which also suggests that myristate is the preferred substrate for VSG myristoylation, was performed in medium containing serum albumin (which binds myristate with approx. 10-fold greater affinity than M-LPC [34,35]) as the only serum protein.

From these data, we propose the following model for M-LPC utilization and GPI myristoylation by African trypanosomes (Figure 6). M-LPC is either hydrolysed by a membrane-associated phospholipase or acylated by acyl-CoA to give PC. Our model to this point is similar to that proposed by Bowes et al. [15], which described the general metabolism of LPC by trypanosomes. However, extracellular myristate is produced by M-LPC hydrolysis and becomes available for the biosynthesis of GPIs or other cellular lipids after uptake and myristoyl-CoA formation. In the presence of both extracellular M-LPC and extracellular myristic acid, myristate produced by M-LPC hydrolysis would be diluted by the pool of pre-existing extracellular

free myristate. Thus serum inhibited VSG myristoylation from [^3H]M-LPC more efficiently than from [^3H]myristate (Figure 5), and non-radioactive myristate inhibited the myristoylation of VSG in serum-free medium from [^3H]myristate with greater efficiency than non-radioactive M-LPC. We predict that under physiological conditions, where myristate and M-LPC are present at approximately equal concentrations in the plasma of infected mammals, trypanosomes would show a kinetic preference for free myristate over M-LPC as a donor for GPI myristoylation.

We have previously demonstrated that analogues of myristic acid are selectively toxic to *T. brucei* in culture [9,10]. Although the mechanism of toxicity is not yet known, we hypothesize that the analogues either interfere with the mechanism of GPI myristoylation or they affect the function of the GPI anchor on VSG. We expect that myristate analogues would be more toxic than their LPC counterparts in serum-rich medium if GPIs were indeed the cellular target for the trypanocidal action of these compounds. Despite this, pharmacokinetic parameters could favour the LPC derivatives. For example, LPC is soluble in water and could present fewer administration problems. Also, the metabolism of these compounds in an infected mammal may differ, which could have a major bearing on drug potency. Studies on the trypanocidal activity of myristate analogue-LPC derivatives are in progress.

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