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Neutral sphingomyelinase activity dependent on Mg^{2+} and anionic phospholipids in the intraerythrocytic malaria parasite *Plasmodium falciparum*

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Sphingolipid metabolism and metabolites are important in various cellular events in eukaryotes. However, little is known about their function in plasmodial parasites. Here we demonstrate that neutral sphingomyelinase (SMase) involved in the sphingomyelin (SM) catabolism is retained by the intraerythrocytic parasite *Plasmodium falciparum*. When assayed in a neutral pH buffer supplemented with Mg^{2+} and phosphatidylserine, an activity for the release of the phosphocholine group from SM was detected in parasite-infected, but not in uninfected, erythrocyte ghosts. The SMase activity in the parasite-infected erythrocyte ghosts was enhanced markedly by anionic phospholipids including unsaturated but not saturated phosphatidylserine. Mn^{2+} could not substitute for Mg^{2+} to activate SMase in parasite-infected erythrocyte ghosts, whereas both Mn^{2+} and Mg^{2+} activated mammalian neutral SMase. The specific activity level of SMase was higher in isolated parasites than in infected erythrocyte ghosts; further fractionation of lysates of the isolated parasites showed that the activity was bound largely to the membrane fraction of the parasites. The plasmodial SMase seemed not to hydrolyse phosphatidylcholine or phosphatidylinositol. The plasmodial SMase, but not SM synthase, was sensitive to scyphostatin, an inhibitor of mammalian neutral SMase, indicating that the plasmodial activities for SM hydrolysis and SM synthesis are mediated by different catalysts. Our finding that the malaria parasites possess SMase activity might explain why the parasites seem to have an SM synthase activity but no activity to synthesize ceramide *de novo*.

Key words: ceramide, phosphatidylserine, scyphostatin, sphingomyelin.

INTRODUCTION

Malaria, from which over 200 million people suffer worldwide, is caused by the protozoan *Plasmodium* genus. Among four *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*) infectious to humans, *P. falciparum* causes the most deadly form of the disease. The parasites proliferate asexually in the host erythrocytes. Intraerythrocytic development and proliferation of the parasites is coupled with the production of membranes including not only organelle membranes of proliferating parasites but also the tubovesicular membrane network in the cytoplasm of infected erythrocytes [1,2]. For membrane production, the parasites synthesize various lipid types *de novo* but also utilize extracellular lipids [1,3,4].

Sphingolipids are widely, if not ubiquitously, distributed in eukaryotes [5]. Sphingolipids have been demonstrated to be essential in the growth of yeast and mammalian cells [6–8]. Sphingolipid biosynthesis is initiated by condensation of L-serine with palmitoyl-CoA to generate 3-oxodihydrosphingosine, which is then converted to dihydrosphingosine. Dihydrosphingosine is N-acylated to form dihydroceramide, which is desaturated to produce ceramide, the common metabolic precursor for sphingomyelin (SM) and glycosphingolipids [9]. SM is the most abundant of the mammalian sphingolipids; the degradation of SM to produce ceramide seems to be responsible for the modulation of various cellular events including proliferation, differentiation and apoptosis [10–12]. In mammalian cells there are two well-known types of sphingomyelinase (SMase), the enzyme catalysing the hydrolysis of SM to ceramide and phosphocholine: one is termed acid SMase and has an optimum pH of approx. 4.8 [13]. The acid SMase can be subclassified into two isoforms, an endosomal/lysosomal acid SMase and a secretory Zn^{2+}-dependent SMase, although these two isoforms are encoded by the same gene [14]. The other type of mammalian SMase, termed neutral SMase, is a membrane-bound and Mg^{2+}-dependent SMase, and has an optimum pH of approx. 7.5 [13]. It has recently been shown that the neutral SMase is stimulated by phosphatidylserine (PtdSer), an anionic phospholipid [15].

SM is also associated with the plasmodial parasites [1,16]. When incubated with intact parasite-infected erythrocytes, radioactive short-chain ceramide and a fluorescent ceramide analogue are converted metabolically to their SM counterparts [17–19], suggesting that the parasites have an activity for synthesizing SM. However, metabolic labelling of parasite-infected erythrocytes with L-[^{14}C]serine or [^{14}C]palmitate has so far failed to detect any sphingolipid synthesis *de novo* [4,20], suggesting that infected erythrocytes have no activity for the synthesis *de novo* of ceramide, the metabolic precursor of SM. A possible mechanism that would account for this paradox is that erythrocyte-derived SM is degraded to provide the parasites with ceramide. However, it remains unknown whether the parasite-infected erythrocytes have SMase activity. Here we demonstrate for the first time that the intraerythrocytic parasite *P. falciparum* retains SMase activity.

Abbreviations used: C_6-NBD-ceramide, 6-[*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-D-*erythro*-sphingosine; GM3, *N*-acetylneuramyl lactosylceramide; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PPMP, D,L-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-propan-1-ol; PtdSer, phosphatidylserine; SM, sphingomyelin; SMase, sphingomyelinase.

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MATERIALS AND METHODS

Materials

Bovine brain SM, PtdSer, egg-yolk phosphatidylethanolamine (PtdEtn), soya-bean phosphatidylinositol, bovine heart cardiolipin, semi-synthetic phosphatidylglycerol and phosphatidic acid were purchased from Sigma. Egg-yolk phosphatidylcholine (PtdCho) and synthetic PtdSer were from Avanti Polar Lipids; bovine buttermilk *N*-acetylneuramyl lactosylceramide (GM3) and D,L-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-propan-1-ol (PPMP) were from Matreya. [*choline-methyl*-^{14}C]SM (55 mCi/mmol), [*choline-methyl*-^{14}C]PtdCho L-α-dipalmitoyl (55 mCi/mmol) and [4,5-^{3}H]dihydrosphingosine (50 Ci/mmol) were purchased from American Radiolabelled Chemicals; L-α-[*myo-inositol*-2-^{3}H(N)]phosphatidylinositol (11 Ci/mmol) was from Dupont NEN. 6-[*N*-(7-Nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-D-*erythro*-sphingosine (C_6-NBD-ceramide) was from Molecular Probes, Triton X-100 was from Pierce, and β-octylglucoside was from Wako Chemicals. Scyphostatin was provided by Dr Futoshi Nara (Sankyo Co., Tokyo, Japan) [21,22].

Cell culture

P. falciparum Honduras 1 strain (no. 1-CDC), which was generously provided by Dr J. Inselburg [23], was routinely maintained by the method of Trager and Jensen [24] with a minor modification. In brief, parasite cells were cultivated in a basic medium [RPMI 1640 (Life Technologies) containing 25 mM Hepes/NaOH, pH 7.2, 3.4 mM L-glutamine, 23.8 mM $NaHCO_3$, 50 μg/ml hypoxanthine and 25 μg/ml gentamicin] supplemented with 10 % (v/v) O-type human serum and 3 % haematocrit O-type human erythrocytes at 37 °C under $CO_2/O_2/N_2$ (1:1:18) with an $N_2/O_2/CO_2$ incubator (BNP-110; Tabai Espec Co.). For the preparation of enzyme sources, the parasite cells synchronized to the ring stage with 5 % (w/v) D-sorbitol [25] and then diluted to approx. 0.15 % parasitaemia were cultivated in the basic medium supplemented with 60 or 90 μM lipid-rich BSA for 94 h. The lipid-rich BSA stock solution used was prepared by dissolving Albumax I™ (Life Technologies) powder in Dulbecco's PBS (Sigma) at 600 μM; it was stored at 4 °C until use. The medium was replaced at 48, 72 and 84 h with fresh medium containing 60, 90 and 90 μM lipid-rich BSA respectively. The final parasitaemia under these culture conditions was 10–15 %. To ensure that the infected and uninfected cell suspensions were comparable, the uninfected erythrocytes were incubated in basic medium supplemented with 60 μM lipid-rich BSA for 94 h without a medium change.

Preparation of enzyme sources

HSI buffer consisted of 10 mM Hepes/NaOH buffer, pH 7.5, 250 mM sucrose and a protein inhibitor cocktail [one tablet of Complete™ Protease Inhibitor (Boehringer Mannheim) per 50 ml]. All manipulations were performed at 4 °C or on ice unless noted otherwise.

For the preparation of *P. falciparum*-infected erythrocyte ghosts, erythrocytes (equivalent to 12 ml of packed cells) were harvested by centrifugation at 520 ***g*** for 5 min. After being washed three times with 120 ml of PBS, the erythrocyte pellet was diluted slowly with 240 ml (20-fold the pellet volume) of deionized water with gentle stirring; the resulting suspension was left for 30 min. The suspension was then centrifuged at 75600 ***g*** for 30 min. The precipitate was suspended in 90 ml of 20 mM Hepes/NaOH buffer, pH 7.5, and centrifuged at 75600 ***g*** for 30 min to wash the erythrocyte ghosts. This washing step was repeated once. The erythrocyte ghosts were then washed with 90 ml of HSI buffer. The washed precipitate as parasite-infected erythrocyte ghosts was suspended in 6 ml of HSI buffer and stored at −80 °C until use. Similarly, uninfected erythrocyte ghosts were prepared from the uninfected erythrocyte culture as described above. It should be noted that the specific activity of neutral SMase in parasite-infected erythrocyte ghosts varied, paralleling the final parasitaemia of parasite cultures.

For the isolation of parasites, erythrocytes (equivalent to 16 ml of packed cells) with approx. 10 % parasitaemia were harvested and washed as described above. The infected erythrocyte pellet was gently suspended in 160 ml (10-fold pellet volume) of PBS containing 0.075 % saponin (Sigma). After incubation at room temperature for 5 min, the suspension was centrifuged at 8400 ***g*** for 10 min; the precipitate was washed three times with 240 ml of PBS. The washed precipitate as isolated parasites was suspended in 1.2 ml of HIS buffer and stored at −80 °C until use. If necessary, the isolated parasites (1.5 mg/ml protein in HSI buffer) were lysed by sonication three times for 5 s at 1 min intervals with a probe-type sonicator. After centrifugation of the lysates at 10^5 ***g*** for 1 h, the supernatant and precipitate fractions were separated; the precipitate fraction as parasite membranes was suspended in HSI buffer with a syringe equipped with a 26-gauge needle.

For the preparation of bovine brain membranes, a bovine brain obtained from a local slaughterhouse was minced with scissors and homogenized in 5 vol. of HES buffer (50 mM Hepes/NaOH buffer, pH 7.5, containing 5 mM EDTA and 0.25 M sucrose] with a Polytron™ (Brinkman). After centrifugation of the homogenates at 18000 ***g*** for 15 min, the supernatant was centrifuged at 100000 ***g*** for 1 h. The precipitate was resuspended in HES buffer and centrifuged at 100000 ***g*** for 1 h to wash the membranes. This washing step was repeated twice. The washed precipitates as bovine brain membranes were suspended in HES buffer at a concentration of approx. 10 mg/ml protein and stored at −80 °C until use.

In all preparations, protein concentrations were determined with the Pierce bicinchoninic acid protein assay kit with BSA as the standard.

Enzyme assays

Assays of acid and neutral SMase activities were performed by the modified method of Schütze and Krönke [26]. To prepare a stock solution of 50 μM radioactive SM substrate, 13.5 μl (25 nmol) of [*choline-methyl*-^{14}C]SM (55 mCi/mmol, 1 mCi/ml in toluene) was placed in a glass tube and the organic solvent was removed under vacuum. The dried [^{14}C]SM was solubilized in 500 μl of 1 % (w/v) β-octylglucoside by brief sonication with a bath-type sonicator. For the standard assay of Mg^{2+}- and PtdSer-dependent neutral SMase activity, 10 μl of deionized water, 10 μl of 50 μM [*choline-methyl*-^{14}C]SM in 1 % (w/v) β-octylglucoside, 10 μl of 5 mM bovine brain PtdSer suspension and 10 μl of 250 mM Hepes/NaOH buffer, pH 7.5, containing 50 mM $MgCl_2$ and 0.5 % (v/v) Triton X-100 were added to a safe-lock 1.5 ml tube (Eppendorf). The reaction was started by the addition of 10 μl of enzyme source (0.04–20 mg/ml protein) to the tube. For enzyme-free controls, 10 μl of HSI buffer was added instead of the enzyme source. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 0.8 ml of chloroform/methanol (2:1, v/v) to the tube. After the addition of 250 μl of deionized water, the tube was mixed vigorously and centrifuged (10000 ***g***, 5 min, room temperature). A 400 μl portion of the aqueous upper phase was transferred to a scintillation vial;

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the radioactivity of the sample in 2 ml of a scintillation cocktail (ACS-II®; Amersham Pharmacia Biotech) was measured with a scintillation counter. Background radioactivity from the enzyme-free controls was subtracted from the radioactivity of each sample. Taking account of the aqueous upper phase with a total volume of 567 μl, the measured radioactivity was multiplied by a factor of 567/400 for correction. A stock suspension of 5 mM PtdSer was prepared as follows: 1.2 ml of PtdSer (10 mg/ml in chloroform) was placed in a thick glass tube; the organic solvent in the tube was completely removed under a stream of nitrogen and subsequently under vacuum. After the addition of 3 ml of deionized water to the tube, PtdSer was dispersed thoroughly with a probe-type sonicator and used for assay. If necessary, suspensions of other phospholipid types were prepared similarly.

For the assay of acid SMase activity, 20 μl of deionized water, 10 μl of 50 μM [*choline-methyl*-^{14}C]SM in 1% (w/v) β-octyl-glucoside and 10 μl of 0.5 M sodium acetate buffer, pH 4.8, containing 0.5% (v/v) Triton X-100 and, if necessary, 50 mM $ZnCl_2$ were added to a 1.5 ml tube. The initiation and termination of the reaction, and the determination of the water-soluble radioactivity released from [*choline-methyl*-^{14}C]SM, proceeded as described above.

SM synthase activity was determined in 50 mM Hepes/NaOH buffer containing 5 mM EDTA and 10 μM C_6-NBD-ceramide as described previously [27], except that parasite membranes (0.2 mg/ml protein in the reaction mixture) were used instead of Chinese hamster ovary cell membranes as the enzyme source.

Dihydrosphingosine-N-acyltransferase activity was determined by a modification of a previous method [28]. In brief, the enzyme source (100 μg of protein) was incubated in 200 μl of 50 mM Hepes/NaOH buffer, pH 7.5, containing 85 nM [4,5-^{3}H]dihydrosphingosine (50 Ci/mmol), 50 μM palmitoyl-CoA and 0.5 mM dithiothreitol at 37 °C for 30 min. Lipids were extracted from the sample and separated by TLC with chloroform/methanol/acetic acid (94:5:5, v/v) as solvent. Radioactive lipids on the TLC plate were detected by image analysis with BAS-1800 (Fuji Film Co.). After the TLC plate had been scraped, the radioactivity of the dihydroceramide produced was determined in the ACS-II® cocktail by scintillation counting.

RESULTS

SMase activity in *P. falciparum*-infected human erythrocytes

To examine whether the malaria parasite *P. falciparum* had SMase activity, we measured SM hydrolysis in ghosts of human erythrocytes infected or uninfected by the parasites, under reaction conditions optimal for the detection of mammalian acid and neutral SMase activities. However, neither parasite-infected nor uninfected erythrocyte ghosts showed any appreciable activity under acidic conditions regardless of the presence of 10 mM $ZnCl_2$ (Table 1). At neutral pH, no activity in uninfected erythrocyte ghosts and only very weak activity in parasite-infected erythrocyte ghosts was detected in the absence of exogenous PtdSer (Table 1). However, the addition of 1 mM bovine brain PtdSer to the neutral SMase assay mixture markedly enhanced the activity for SM hydrolysis in parasite-infected erythrocyte ghosts, whereas no activity for SM hydrolysis was observed in uninfected erythrocyte ghosts even when PtdSer was supplied (Table 1).

Phospholipase C and D are defined as activities to release the phosphoryl base moiety and the base moiety respectively from phospholipid substrates. We determined which phospholipase type the SM hydrolytic activity in parasite-infected erythrocyte ghosts was. Water-soluble radioactivity liberated from [*choline-methyl*-^{14}C]SM by the reaction with parasite-infected erythrocyte ghosts co-migrated with a standard phosphocholine, but not with choline, in TLC, similarly to when bovine brain membranes were used as an enzyme source of mammalian neutral SMase (Figure 1). We therefore assigned the activity specifically detected in parasite-infected erythrocyte ghosts to an SMase of phospholipase C type.

The time course of the SMase activity of parasite-infected erythrocyte ghosts in the presence of 1 mM PtdSer was linear for at least up to 30 min; this activity was also directly proportional to the amounts of proteins up to 100 μg (results not shown). The

Table 1 SM hydrolysis in *P. falciparum*-infected or uninfected erythrocyte ghosts under various reaction conditions

P. falciparum-infected or uninfected erythrocyte ghosts (100 μg of protein) were incubated with [*choline-methyl*-^{14}C]SM in 0.1 M sodium acetate buffer, pH 4.8, containing 0.1% (v/v) Triton X-100 in the presence or absence of 10 mM $ZnCl_2$, or in 50 mM Hepes/NaOH buffer, pH 7.5, containing 0.1% (v/v) Triton X-100 and 10 mM $MgCl_2$ in the presence or absence of 1 mM bovine brain PtdSer, at 37 °C for 30 min. The water-soluble radioactivity released from [^{14}C]SM was measured as described in the Materials and methods section. Results are means ± S.D. for triplicate experiments.

	SM hydrolysed (pmol/h per mg of protein)	
Assay conditions	Infected	Uninfected
pH 4.8	< 5	< 5
pH 4.8, 10 mM $ZnCl_2$	< 5	< 5
pH 7.5, 10 mM $MgCl_2$	6.4 ± 1.1	< 5
pH 7.5, 10 mM $MgCl_2$, 1 mM PtdSer	331 ± 8	< 5

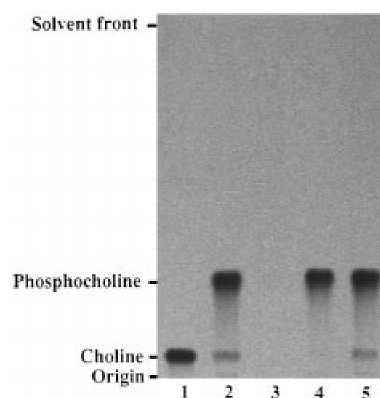

Figure 1 Release of phosphocholine from SM by incubation with *P. falciparum*-infected erythrocyte ghosts

P. falciparum-infected human erythrocyte ghosts (100 μg of protein) or bovine brain membranes (10 μg of protein) were incubated in 50 mM Hepes/NaOH buffer, pH 7.5, containing 0.1% (v/v) Triton X-100, 10 mM $MgCl_2$, 1 mM bovine brain PtdSer and 10 μM [*choline-methyl*-^{14}C]SM at 37 °C for 1 h. After termination of the reaction by the addition of chloroform/methanol, aqueous and organic solvent fractions were separated as described in the Materials and methods section. For standards (lanes 1 and 2), [*methyl*-^{14}C]choline or [*methyl*-^{14}C]phosphocholine (Amersham Pharmacia Biotech) was added to the aqueous fraction separated from the reaction buffer without the enzyme source and [^{14}C]SM. After the aqueous fraction had been dried under vacuum, the sample was dissolved in 50 μl of methanol, applied to a TLC plate (silica gel 60; Merck) and developed with a solvent consisting of methanol/0.5% NaCl/28% (w/v) ammonia (100:100:2, v/v). The radioactive image of the plate detected with an image analyser is shown. Lane 1, [*methyl*-^{14}C]choline standard; lane 2, [*methyl*-^{14}C]phosphocholine standard; lane 3, no enzyme source; lane 4, parasite-infected human erythrocyte ghosts; lane 5, bovine brain membranes.

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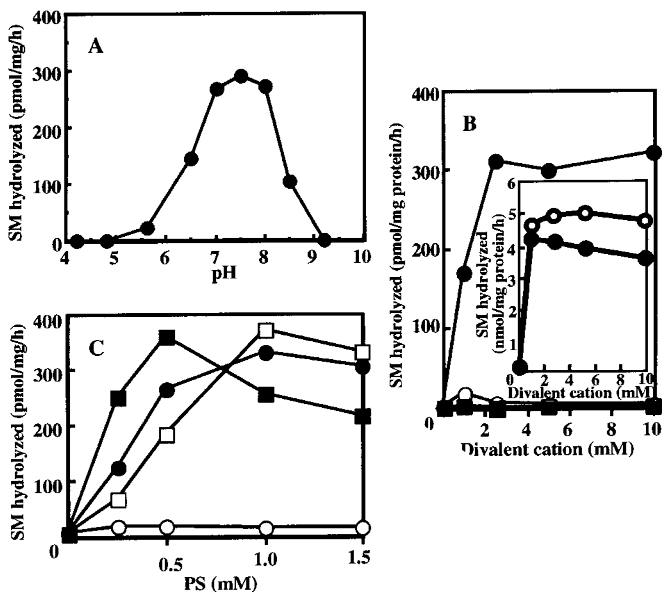

Figure 2 SMase activity in *P. falciparum*-infected erythrocyte ghosts

(**A**) pH-dependence of the activity. Parasite-infected erythrocyte ghosts (100 μg of protein) were incubated in various buffers {0.1 M sodium acetate buffers for pH 4.2–9.2; 0.1 M 1,3-bis[tris(hydroxylmethyl)methylamino]propane/HCl buffers for pH 6.5–9.2} containing 0.1 % (v/v) Triton X-100, 10 mM $MgCl_2$, 1 mM bovine brain PtdSer and 10 μM [*choline-methyl*-^{14}C]SM at 37 °C for 30 min. Means for duplicate experiments are shown. (**B**) Dependence of neutral SMase activity of *P. falciparum*-infected erythrocyte ghosts on bivalent cations. *P. falciparum*-infected human erythrocyte ghosts (100 μg of protein) were incubated in 50 mM Hepes/NaOH buffer, pH 7.5, containing 0.1 % (v/v) Triton X-100, 1 mM bovine brain PtdSer and 10 μM [*choline-methyl*-^{14}C]SM and various concentrations of bivalent cations as chloride salts at 37 °C for various durations. Water-soluble radioactivity released from [^{14}C]SM was measured as described in the Materials and methods section. Inset, bovine brain membranes (100 μg of protein) were used as the enzyme source. Symbols: ●, $MgCl_2$; ○, $MnCl_2$; ■, $CaCl_2$. Means for triplicate experiments are shown. (**C**) Dependence of the activity on PtdSer. Parasite-infected erythrocyte ghosts (100 μg of protein) were incubated in the reaction buffer containing various concentrations of bovine brain PtdSer (●), dipalmitoyl PtdSer (○), dioleoyl PtdSer (■) and *sn*-1-palmitoyl-2-oleoyl PtdSer (□) at 37 °C for 30 min. Means for triplicate experiments are shown.

optimum pH for this activity was between pH 7.0 and 8.0 (Figure 2A); the apparent K_m for SM was approx. 100 μM (see below).

Dependence of the SMase activity of infected erythrocytes on bivalent cations

The neutral SMase activity in parasite-infected erythrocyte ghosts was highly dependent on Mg^{2+}, with an ED_{50} of approx. 1 mM (Figure 2B). Ca^{2+} was ineffective (Figure 2B). Interestingly, Mn^{2+} could not substitute for Mg^{2+} in activating the SMase activity in parasite-infected erythrocyte ghosts (Figure 2B), whereas Mn^{2+} and also Mg^{2+} activated the bovine brain neutral SMase (Figure 2B, inset). The latter observation was consistent with previous studies on mammalian neutral SMase [15,29–31].

Dependence of the SMase activity of infected erythrocytes on phospholipids

The dependence of the neutral SMase activity of parasite-infected erythrocyte ghosts on bovine brain PtdSer reached saturation at approx. 1 mM (Figure 2C, ●). The major molecular species of bovine brain PtdSer is *sn*-1-stearoyl-2-oleoyl PtdSer [32]. For examination of the dependence of the activity on molecular species of PtdSer, various forms of chemically synthesized PtdSer were used in place of bovine brain PtdSer in the assay. Dioleoyl PtdSer and *sn*-1-palmitoyl-2-oleoyl PtdSer were similarly effective, whereas dipalmitoyl PtdSer was almost ineffective, compared with natural PtdSer derived from the brain (Figure 2C).

We also examined whether phospholipid types other than PtdSer enhanced the neutral SMase activity of parasite-infected erythrocyte ghosts. As shown in Table 2, neither PtdCho nor PtdEtn enhanced the activity at all. In contrast, 1 mM phosphatidylglycerol, phosphatidylinositol, phosphatidic acid and cardiolipin each substantially enhanced the SMase activity to 20–80 % of that observed in the presence of 1 mM bovine brain PtdSer. Other anionic amphipathic compounds, including GM3 ganglioside, cholic acid and bacterial lipopolysaccharide, had little or no effect on the activity. Similarly, L-serine (1 mM) and polyanionic compounds (0.2 mg/ml) including heparin and dextran-sulfate were ineffective (results not shown). It was unlikely that the failure of PtdCho and PtdEtn to enhance the neutral SMase activity was due to a difference in the fatty acid composition between these ineffective phospholipids and PtdSer because the major molecular species of natural PtdCho and PtdEtn that we used were *sn*-1-palmitoyl-2-oleoyl PtdCho and *sn*-1-stearoyl-2-oleoyl PtdEtn respectively [32]. Thus both the anionic head group and the unsaturated acyl group of phospholipids seemed to have key roles in activating the neutral SMase of parasite-infected erythrocyte ghosts.

Table 2 SMase activity of *P. falciparum*-infected erythrocyte ghosts requires anionic phospholipids

P. falciparum-infected erythrocyte ghosts (100 μg of protein) were incubated with [*choline-methyl*-^{14}C]SM in 50 mM Hepes/NaOH buffer, pH 7.5, containing 0.1 % (v/v) Triton X-100, 10 mM $MgCl_2$ and the indicated additions (1 mM) or 0.2 mg/ml lipopolysaccharide (from *Escherichia coli* Serotype 0111 :B4; Sigma) at 37 °C for 30 min. The water-soluble radioactivity released from [^{14}C]SM was then measured as described in the Materials and methods section. The amounts of hydrolysed SM are shown as percentages ($\pm$S.D.) of the mean level in the presence of 1 mM bovine brain PtdSer, and are means $\pm$ S.D. for triplicate experiments.

Addition	Relative SMase activity (%)
None	< 3
PtdSer	100 ± 6
PtdCho	< 3
PtdEtn	< 3
Phosphatidylinositol	53 ± 5
Phosphatidylglycerol	79 ± 4
Phosphatidic acid	33 ± 1
Cardiolipin	21 ± 0
GM3 ganglioside	< 3
Cholic acid	5 ± 0
Lipopolysaccharide	< 3

Enrichment of the neutral SMase in the membrane fraction of the parasites

We next examined whether the neutral SMase activity detected in parasite-infected erythrocyte ghosts was physically associated with the parasites. Infected erythrocyte ghosts (which contain intact parasites in addition to the plasma membrane of the host erythrocytes) and isolated parasites were prepared from the same batch of parasite culture; the specific activity of SMase was compared between the two preparations. The specific activities of infected erythrocyte ghosts and isolated parasites were 569 ± 31 and 2097 ± 73 pmol/h per mg of protein (means $\pm$ S.D.; $n = 3$) respectively. For further fractionation, isolated parasites were lysed by sonication and, after high-speed centrifugation of the lysate, supernatant and precipitate fractions were separated.

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Table 3 Enrichment of SMase activity in the membrane fraction of isolated parasites

After lysis of the isolated parasites by sonication, the lysates were centrifuged (10^5 ***g***, 4 °C, 1 h) and the supernatant and precipitate fractions were separated as described in the Materials and methods section. Each fraction was assayed for SMase activity and protein concentration. Distributions of protein and SMase activity to each fraction are shown as percentages (means ± S.D.) of the mean values of the lysate fraction for triplicate experiments; specific activities of SMase are means ± S.D. for triplicate experiments.

Fraction	SMase activity (% of total lysate)	Protein (% of total lysate)	Specific activity of SMase (pmol/h per mg of protein)
Lysate	100 ± 1	100 ± 1	2100 ± 70
Supernatant	10 ± 0	29 ± 1	910 ± 30
Precipitate	101 ± 1	74 ± 1	3460 ± 40

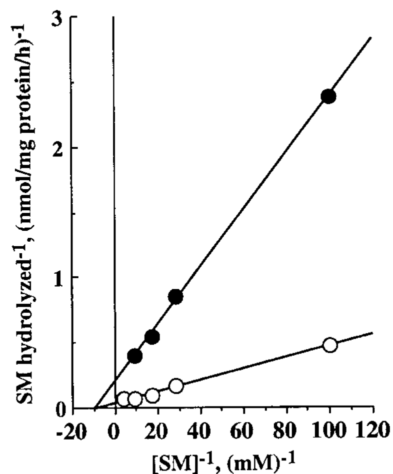

Figure 3 Double-reciprocal plot analysis of neutral SMase activity in *P. falciparum*-infected erythrocyte ghosts and parasite membranes

P. falciparum-infected human erythrocyte ghosts (100 μg of protein) or parasite membranes (20 μg of protein) were incubated in 50 mM Hepes/NaOH buffer, pH 7.5, containing 0.1 % (v/v) Triton X-100, 10 mM $MgCl_2$, 1 mM bovine brain PtdSer and various concentrations of [*choline-methyl*-^{14}C]SM at 37 °C for 30 min. Water-soluble radioactivity released from [^{14}C]SM was determined as described in the Materials and methods section. When [^{14}C]SM diluted with non-radioactive bovine SM was used as the substrate, the amount of SM hydrolysed in the reaction was corrected for dilution. Symbols: ○, parasite membranes; ●, parasite-infected erythrocyte ghosts. Note that the K_m and V_{max} values estimated from these plots are the apparent values because the contribution of SM endogenously associated with the enzyme sources to the substrate concentrations was neglected in this analysis.

Most of the SMase activity in the lysate was recovered in the precipitate fraction; the specific activity of SMase was enriched in the precipitate but not in the supernatant in comparison with the activity in the lysates (Table 3). The total activity recovered in the fractionation experiment was approx. 10 % higher than the original activity of the lysates. This might have been due to the separation of an unknown cytosolic factor affecting the SMase activity from the membrane fraction. Nevertheless, the apparent V_{max} values of the activity in parasite-infected erythrocyte ghosts and the parasite membranes were 4.9 and 29 nmol of SM hydrolysed/h per mg of protein respectively, whereas there was no appreciable difference in the apparent K_m values (approx. 100 μM) for SM between the two enzyme sources (Figure 3), demonstrating that the SMase activity enriched in the parasite membranes reflected the activity detected in parasite-infected erythrocyte ghosts. These results indicate that the neutral SMase activity detected specifically in parasite-infected erythrocyte ghosts is bound largely to parasite-associated membranes; we therefore refer to this activity as the plasmodial SMase from here onwards.

Substrate specificity of the plasmodial SMase

To elucidate the phospholipid substrate specificity of the plasmodial SMase, we examined whether the isolated parasites showed hydrolytic activity towards PtdCho or phosphatidylinositol. Isolated parasites (24 μg of protein) were incubated with 10 μM [*choline-methyl*-^{14}C]SM (55 mCi/mmol), [*choline-methyl*-^{14}C]PtdCho (55 mCi/mmol) or [myo-*inositol*-2-^{3}H]phosphatidylinositol (400 mCi/mmol) in 50 mM Hepes/NaOH buffer, pH 7.5, containing 0.1 % (v/v) Triton X-100, 10 mM $MgCl_2$ and 1 mM PtdSer at 37 °C for 30 min. Under these assay conditions, hydrolytic activities towards SM, PtdCho and phosphatidylinositol were 1775 ± 57, 143 ± 9 and less than 5 pmol/h per mg of protein (means ± S.D.; $n = 3$) respectively, indicating a high substrate specificity of the plasmodial SMase. Note that the weak activity for PtdCho hydrolysis of the isolated parasites was probably due to contamination by erythrocyte membranes of the parasite preparation, because the specific activity of PtdCho hydrolysis was higher in parasite-infected and uninfected erythrocyte ghosts than in the isolated parasites (results not shown).

Effects of scyphostatin and PPMP on activities of the plasmodial SMase and SM synthase

Scyphostatin is a potent inhibitor of mammalian neutral SMase but not acid SMase or bacterial SMases [21,22]. The SMase activity associated with the parasite membranes showed sensitivity to scyphostatin with an IC_{50} of approx. 5 μM, which is similar to the neutral SMase activity of bovine brain membranes (Figure 4A). For a test of the specificity of this drug, the effect of scyphostatin on the parasite-associated SM synthase activity was examined by using C_6-NBD-ceramide as the enzyme substrate. The conversion of C_6-NBD-ceramide into its SM metabolite, C_6-NBD-SM, was not appreciably affected by 33 μM scyphostatin (Figure 4B), at which concentration the plasmodial SMase was almost completely inhibited (Figure 4A). In contrast, the plasmodial SM synthase activity was partly inhibited by 50 μM PPMP (Figure 4D), whereas 50 μM PPMP caused no appreciable inhibition of the plasmodial SMase activity (Figure 4C). These results indicate that the plasmodial neutral SMase and mammalian neutral SMase are similar in sensitivity to scyphostatin; this is evidence that SM hydrolysis and SM synthesis in the parasite-infected erythrocytes are mediated by distinct catalysts.

Analysis of dihydrosphingosine-N-acyltransferase activity in parasite-infected erythrocytes *in vitro*

To examine the levels of activity of dihydrosphingosine-N-acyltransferase, the key enzyme in ceramide synthesis, we com-

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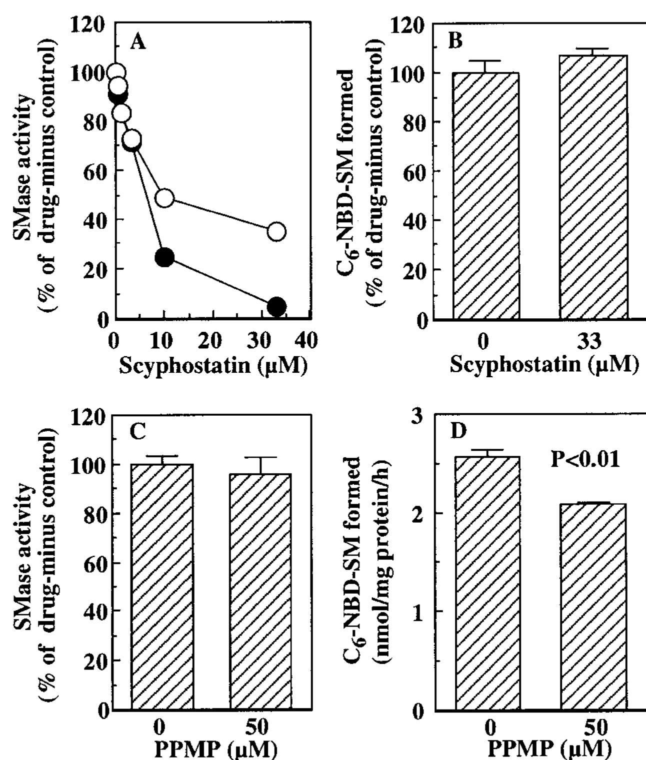

Figure 4 Effects of scyphostatin and PPMP on activities of the plasmodial SMase and SM synthase

(**A**) Parasite membranes (0.4 mg/ml protein) or bovine brain membranes (40 μg/ml protein) were incubated in HSI buffer containing 0.1 % (v/v) Triton X-100 and various concentrations of scyphostatin on ice for 30 min. The concentration of DMSO, the solvent for the drug stock solution, was adjusted to 1 % (v/v) in each sample during incubation. The neutral SMase activity of each sample was then determined. The activity is represented as the percentage of the mean of the drug-free controls for duplicate experiments. Symbols: ●, parasite membranes; ○, bovine brain membranes. (**B**) After incubation of parasite membranes (0.4 mg/ml protein) on ice for 30 min in HSI buffer with or without 33 μM scyphostatin, the SM synthase activity of each sample was determined. The activity is represented as the percentage of the mean of the drug-free control for triplicate experiments. (**C**) After incubation of parasite membranes (0.4 mg/ml protein) on ice for 30 min in HSI buffer containing 0.1 % (v/v) Triton X-100 with or without 50 μM PPMP, the neutral SMase activity of each sample was determined. The activity is represented as the percentage of the mean of the drug-free control for triplicate experiments. (**D**) After incubation of parasite membranes (0.4 mg/ml protein) on ice for 30 min in HSI buffer with or without 50 μM PPMP, the SM synthase activity of each sample was determined. Results in (**B–D**) are means $\pm$ S.D. for triplicate experiments. Statistical analysis was performed with Student's *t* test.

pared the enzyme activity between parasite-infected erythrocyte ghosts, parasite membranes and bovine brain membranes *in vitro*. Dihydrosphingosine-N-acyltransferase activity in parasite-infected erythrocyte ghosts and parasite membranes was below the detectable level (less than 2 pmol of dihydroceramide formed/30 min per mg of protein) under the assay conditions used, whereas bovine brain membranes showed a substantial level of activity (14.3 $\pm$ 0.6 pmol of dihydroceramide formed/30 min per mg of protein), suggesting that parasite-infected erythrocytes had very little or no dihydrosphingosine-N-acyltransferase activity.

DISCUSSION

In the present study we have obtained compelling biochemical evidence that malaria parasite-infected erythrocytes possess SMase activity. Under assay conditions *in vitro*, the SM hydrolytic activity of parasite-infected erythrocyte ghosts requires both Mg^{2+} and unsaturated forms of PtdSer or other anionic phospholipids and is optimal at a neutral pH (Figure 2 and Table 2). This activity catalyses the release of the phosphocholine group from SM (Figure 1), and seems not to hydrolyse other phospholipids, including PtdCho and phosphatidylinositol. We therefore attribute the enzyme activity detected in the parasite-infected erythrocyte ghosts to a Mg^{2+}- and anionic-phospholipid-dependent neutral SMase of phospholipase C type.

The specific activity of Mg^{2+}- and anionic-phospholipid-dependent neutral SMase was higher in isolated parasites than in the parasite-infected erythrocyte ghosts overall; further fractionation of the isolated parasites showed that the activity binds largely to the membrane fraction (Table 3). In addition, the apparent K_m values for SM of the SMase activity in the parasite membrane fraction and parasite-infected erythrocyte ghosts were quite similar (Figure 3). It is therefore likely that the SMase detected in parasite-infected erythrocyte ghosts is a membrane-bound enzyme of the parasites. Uninfected erythrocyte ghosts exhibited no neutral or acid SMase activity; parasite-infected erythrocyte ghosts contained no acid SMase activity (Table 1). It is not surprising that uninfected erythrocyte ghosts showed no activity of acid or neutral SMase because human mature erythrocytes are incapable of synthesizing proteins *de novo* and are also devoid of most of the intracellular organelles including endosome/lysosomes. We suggest that the plasmodial SMase activity demonstrated in this study is not due to the activation of a latent erythrocytic SMase via the parasite invasion and that the parasites themselves express the SMase.

The plasmodial neutral SMase resembles mammalian neutral SMase in several ways. Both enzymes are Mg^{2+}-dependent and membrane-bound SMases, being optimally activated by PtdSer among various anionic phospholipid types (Figure 2B, C and Table 2) [15], and are inhibited by scyphostatin with similar IC_{50} values (Figure 4A) [22]. Nevertheless, there is an interesting difference between them: mammalian neutral SMase but not the plasmodial neutral SMase is activated by Mn^{2+} (Figure 2B). Several enzymic characteristics of the plasmodial SMase are also different from those of the bacterial SMase secreted from *Bacillus cereus*: the bacterial SMase activity is activated by Mn^{2+} as well as Mg^{2+} [33] and is resistant to scyphostatin [22]. In addition, *B. cereus* SMase functions without anionic phospholipids *in vitro* [33], although it is unknown whether anionic phospholipids enhance the bacterial SMase activity. There is a high structural similarity between *B. cereus*, *Staphylococcus aureus* and *Leptospira interrogans* SMases [34–36] and also a significant similarity between these bacterial SMases and a mammalian SMase [31]. Elucidation of the structural similarity or diversity in mammalian, plasmodial and bacterial SMases should await the identification of a plasmodial gene encoding the neutral SMase.

It has been suggested that malaria parasites possess at least two isoforms of SM synthase, on the basis of observations that labelling with C_6-NBD-ceramide and radioactive short-chain ceramide produces their SM counterparts in parasite-infected erythrocytes but not in uninfected erythrocytes [17,18], and that part of the SM synthase activity is highly sensitive to the drug PPMP but another part is not [19]. Considering that the reaction to transfer phosphocholine from PtdCho to ceramide by SM synthase chemically resembles the reverse reaction of SM hydrolysis, one might imagine that the observed SM synthase activity was due to a possible side reaction of SMase. However, the plasmodial SMase is resistant to PPMP, at least up to 50 μM (Figure 4C), whereas the plasmodial SM synthase activity was partly inhibited by 50 μM PPMP (Figure 4D). Moreover, the plasmodial SMase activity is highly sensitive to scyphostatin,

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whereas the plasmodial SM synthase activity is resistant (Figure 4A,B). These results demonstrate that the plasmodial SM synthase is a catalyst distinct from the plasmodial SMase.

Although the malaria parasites have SM synthase activity [17,18], parasite-infected erythrocytes seem to have no activity for the synthesis *de novo* of ceramide, the metabolic precursor of SM [4,20] (see also the Results section). Our finding that the parasites have an SMase activity provides a possible mechanism to account for this paradox: the parasites might degrade erythrocytic SM by the catalytic action of the plasmodial SMase to produce ceramide, which could be a source for the resynthesis of SM by a plasmodial SM synthase. However, it remains undetermined whether the plasmodial SMase catabolizes host cell SM in intact cells. Part of the parasite-associated SM synthase has been suggested to exist in the tubovesicular membrane [37], which extends from the parasitophorous vacuole membrane surrounding the internalized parasites to the erythrocyte membrane [38]. In addition, various types of lipophilic fluorescent probe have been shown to transfer from the host erythrocyte membrane to the parasitophorous vacuole membrane or tubovesicular membranes [17,39,40], suggesting that parasitized erythrocytes possess the lipid-trafficking activity within them. We therefore speculate that the host-cell SM is accessible to the plasmodial SMase through the tubovesicular membrane structure and that the hydrolysis of host-cell SM mediated by the SMase occurs in the tubovesicular membrane structure, where resynthesis of SM could also occur. Alternatively, a minor part of the plasmodial SMase might be translocated to the erythrocyte membrane for degradation of the host-cell SM. Ceramide has been demonstrated to modulate the proliferation and differentiation of various eukaryotes including mammalian and yeast cells [10–12,41]. By analogy, ceramide produced by parasites might modulate the progression of the cell cycle of the parasites; resynthesis of SM by parasites might have a role in the regulation of the ceramide level. We are currently undertaking a study on the effects of scyphostatin on the proliferation and development of the intraerythrocytic parasites.

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