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Phospholipid biosynthesis in the oyster protozoan parasite, *Perkinsus marinus*

Eric D. Lund, Fu-Lin E. Chu *

Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA

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Abstract

Perkinsus marinus is a protozoan parasite that causes high mortality in its commercially and ecologically important host, the Eastern oyster *Crassostrea virginica*. In order to understand the host–parasite relationship in lipid metabolism, the ability of *P. marinus* to synthesize phospholipids from polar headgroup precursors was investigated. Pulse/chase experiments were conducted using radiolabled serine, choline, ethanolamine and inositol. Timecourse incubations revealed that in vitro cultured *P. marinus* meronts can utilize the cytidine diphosphate-diacylglycerol (CDP-DAG) pathway to synthesize phosphatidylinositol (PI) from inositol and phosphatidylserine (PS) from serine. Serine label was also incorporated into phosphatidylethanolamine (PE), phosphatidylcholine (PC) and lysophosphatidylcholine (LPC). Incubations of *P. marinus* cells with increasing concentrations of radiolabeled serine resulted in more radioactivity recovered in neutral lipids than in polar lipids at the highest substrate concentration tested (344 μM). This suggests that excess serine label was being utilized for fatty acid synthesis and stored as triacylglycerols. Additional incubations were conducted with radiolabeled choline and ethanolamine at concentrations equimolar to the highest serine concentration tested. Ethanolamine label was also incorporated into PE, PS, PC and LPC. Choline label was incorporated into PC. These results suggest the presence of three pathways for de novo synthesis of phospholipids in *P. marinus*: CDP-choline, CDP-ethanolamine and CDP-DAG. At equivalent substrate concentrations (344 μM) the highest incorporation of labeled substrate into total phospholipids was with serine followed by ethanolamine and choline, respectively. *P. marinus* phospholipid biosynthetic capabilities appear to be similar to those of *Plasmodium* and *Trypanosoma* species. © 2002 Published by Elsevier Science B.V.

Keywords: *Crassostrea virginica*; Lipid biosynthesis; Oyster; Oyster parasite; *Perkinsus marinus*; Phospholipids

1. Introduction

Successful replication of protozoan parasites is predicated on their ability to either acquire extracellular and intracellular components from their host or to synthesize them from host-derived precursors. As a parasitic infection progresses within a host the total surface area of parasites increases with each cycle of cell replication. For the infection to proceed the parasites must be able to synthesize new cell membrane components, especially phospholipids which comprise most of the lipid bilayer of the membrane.

The essential nature of phospholipid synthesis and/or acquisition for replication of protozoan parasites has been recognized and exploited for the purposes of treating human diseases caused by these organisms. Molecules that block phosphatidylcholine synthesis either directly [1], or indirectly [2,3] have been shown to inhibit cell replication in vitro in *Trypanosoma cruzi*. As such, further understanding of the pathways utilized by different parasitic protozoans for acquiring, producing and modifying phospholipids should elucidate pathways that differ from their hosts. These novel

Abbreviations: CDP, cytidine diphosphate; CHE, cholesterol; DAG, diacylglycerol; FFA, free fatty acid; GPI, glycosylphosphatidylinositol; HPTLC, high performance thin layer chromatography; LPC, lysophosphatidylcholine; NL, neutral lipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, polar lipid; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglycerol; VSG, variant surface glycoprotein.

* Corresponding author. Tel.: +1-804-684-7349; fax: +1-804-684-7186.

E-mail address: chu@vims.edu (F.-L.E. Chu).

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pathways may prove useful targets for drug development.

Perkinsus marinus is a protozoan parasite that infects the Eastern oyster, *Crassostrea virginica*, along the Atlantic and Gulf coasts. It is one of the two most prevalent parasites of the Eastern oyster and has caused high mortalities in this commercially and ecologically important species since at least the late 1940s [4–7]. Four life stages of *P. marinus* have been described: meront (trophozoite), prezoosporangium (hypnospore), zoosporangium and biflagellated zoospore [8,9]. Three of these life stages, meront, prezoosporangium and biflagellated zoospore, have been demonstrated to be infective via transmission through the water column [10]. The meront stage has been suggested to be the primary life stage for disease transmission and can be found both intercellularly and intracellularly in infected oysters [9,10]. *P. marinus* was originally described as a member of the Phylum Apicomplexan [11,12] based on morphological and ultrastructural analyses. Subsequent genetic analyses suggest that *P. marinus* is more closely phylogenetically related to the dinoflagellates than the apicomplexans [13,14]. Recent advances in isolation and culture techniques have resulted in the ability to culture the meront stage of *P. marinus* axenically in both undefined media [15–17] and defined media [18,19]. Cultured meronts have been shown to remain infective and sufficiently virulent to cause host mortality [15,17,20].

Previous studies on the lipid biosynthetic pathways of *P. marinus* have revealed that this organism has extensive capabilities to synthesize lipids de novo as well as modify lipids obtained from the environment. Using flourescent lipid analogues it has been shown that *P. marinus* meronts are capable of incorporating exogenous free fatty acids (FFA), cholesterol esters (CHE), phosphatidycholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) [21]. Furthermore, incorporated CHE were metabolized to FFA and triacylglycerol (TAG); FFA was metabolized into TAG, dicacylglycerol (DAG) and PC; PE was metabolized into PC, TAG and DAG and PC was metabolized to PE [21]. It has also been shown that *P. marinus* meronts cultured in vitro can synthesize fatty acids and lipid classes de novo during cell proliferation [22]. The synthesis of saturated, monunsaturated and polyunsaturated $n-6$ fatty acids by *P. marinus* has been confirmed using ^{13}C-labeled sodium acetate as a precursor [23].

Although it is known that *P. marinus* is capable of synthesizing phospholipids from non-lipid components of defined media [24], the phospholipid classes produced and the pathway(s) for their synthesis have yet to be investigated. Other parasitic protozoans exhibit a range of capabilities for synthesizing phospholipids. Some, such as *Plasmodium* sp., are capable of making all required polar lipids from precursors [25] whereas others, such as *Giardia lamblia*, must acquire all polar lipids from their host [26].

In order to test its ability to synthesize phospholipids and the pathways that may be utilized for their synthesis, *P. marinus* cells were incubated in vitro with a series of radiolabeled phospholipid precursors. (^{3}H serine, ^{14}C ethanolamine, ^{14}C choline and ^{3}H myo-inositol).

2. Materials and methods

Log phase meronts of *P. marinus* grown in the medium described by LaPeyre et al. [17] were resuspended at a density of 2×10^6 cells per ml in 5 ml of 20 precipitate artificial seawater with 1% (v/v) Gibco lipid concentrate (cholesterol, 450 mg l^{-1}; cod liver oil, 1 g l^{-1}; DL-α-tochopherol acetate 200 mg l^{-1}, Pluronic F-68, 100 g l^{-1}; Tween 80, 2.5 g l^{-1}; Life Technologies Inc., Rockville, MD) containing a single radiolabeled polar headgroup substrate (serine, inositol, choline or ethanolamine; Dupont NEN, Washington DC).

The initial experiment tested the time-dependence of substrate incorporation using ^{3}H serine (87 nM, 22300 μCi μmole^{-1}) and myo-inositol [2-^{3}H(N)] (90 nM, 22.3 μCi μmole^{-1}) at 28 °C for 30 min, 1, 2 and 3 h ($n = 3$). Incubations were conducted in triplicate and terminated by the addition of 0.5 ml methanol. The cells were pelleted via centrifugation at $300 \times g$ for 5 min. Supernatants containing the unmetabolized substrate were removed and the cells were resuspended in 1.5 ml water prior to lipid extraction.

A second experiment tested the concentration-dependence of serine incorporation and compared choline, ethanolamine and serine incorporation at equimolar concentrations. Substrates tested using three replicates each were L-serine [^{3}H (G)] at concentrations of 1, 10, 100 and 344 μM (1700, 170, 17 and 5 μCi μmole^{-1}, respectively), 344 μM choline chloride [methyl-^{14}C] (6 μCi μmole^{-1}) and 344 μM ethanolamine hydrochloride [1, 2-^{14}C] (3 μCi μmole^{-1}). All of these incubations were conducted at 28 °C for 90 min.

Lipids were extracted by the method of Bligh and Dyer [27] and stored in chloroform: methanol (1:1) at −20 °C for no more than 2 months before analysis by high performance thin layer chromatography (HPTLC). Any residual non-metabolized substrate that was not removed with the supernatant was dissolved in the aqueous phase and removed during lipid extraction. Lipid classes were separated using Whatmann LHPK Silica Gel 60A HPTLC plates (Whatmann, Clifton, NJ). All plates were pre-developed in hexane:diethyl ether (1:1) and dried at 100 °C for 2 h prior to use. Samples were dried under a stream of nitrogen, resuspended in 50 μl of chloroform: methanol (1:1) and 10 μl was spotted

on the plate. Between 10–50 μg of non-radiolabeled standards of LPC (lysophospatidylcholine), SM (sphingomyelin), PC (phsophatidylcholine), PS (phosphatidylserine), PI (phosphatidylinositol) and PE (phosphatidylethanolamine) (Sigma, St. Louis, MO) were also spotted on each plate. Plates were developed in methyl acetate: *n*-propanol:chloroform:methanol:0.25% potassium chloride (25:25:25:10:9) for 1 h. This solvent mixture separated phospholipids into various classes. Neutral lipids, including triacylglycerol, free fatty acids and sterol esters, migrated together with the solvent front to the top of the plate. After air drying for 20 min separated lipid classes were visualized using iodine vapor and identified relative to standards (Fig. 1). Silica in lanes from *P. marinus* lipids corresponding to identified phospholipid classes and the neutral lipids was scraped from the glass plate backing into scintillation vials for liquid scintillation counting or into glass vials containing chloroform: methanol (1:1) for further analysis.

Three aliquots of the *P. marinus* culture used for the 90 min serine, ethanolamine and choline assays were archived for subsequent determination of protein content. Cell pellets were freeze dried, lysed with 1 M NaOH at 37 °C overnight, sonicated, then assayed for total protein according to the method of Lowry [28].

The distribution of radiolabel in neutral lipid classes was determined by separating the neutral lipids into individual lipid classes by HPTLC using a different solvent system. Solvent containing the neutral lipids was filtered through a glass fiber filter, dried under a stream of nitrogen and resuspended in 30 μl of chloroform:methanol (1:1). About 10 μl of the neutral lipids was spotted on a HPTLC plate along with neutral lipid standards (triolein, oleic acid and cholesteryloleate) and the plate was developed in hexane:diethyl ether:-formic acid (85:15:0.04) for 40 min. Spots corresponding to each lipid class were visualized (Fig. 1), scraped and quantified by liquid scintillation counting as described above for the phospholipids.

The presence of radiolabel in the fatty acyl components of lipid classes was determined by transmethylating each separated lipid class and measuring isotope incorporation in organic and aqueous fractions. Briefly, lipid fractions recovered from scraped silica with chloroform:methanol (1:1) were filtered through glass fiber filters, then dried under a stream of nitrogen. An aliquot of 250 μl of methanolic boron trifluoride (Supelco, Bellefonte, PA) was added to each sample, vials were sealed, then heated to 100 °C for 30 min. After cooling, 250 μl distilled water and 500 μl hexane were added to each sample. Samples were then vortexed and allowed to form a biphasic solution. The upper phase (hexane) of each sample, containing only the fatty acid methyl esters, was transferred to one scintillation vial and the lower phase (methanol–water) containing the rest of the polar lipid components was transferred to a separate scintillation vial for subsequent liquid scintillation counting.

All results are expressed as mean ± standard deviation. Data from incorporations of 344 μM choline, ethanolamine and serine into phospholipids were subjected to one-way analysis of variance (ANOVA) and multiple comparisons were determined by Tukeys test. Analyses were performed using a MINITAB computer package.

3. Results

3.1. Timecourse incubations

Uptake and incorporation of serine label into phospholipids of *P. marinus* increased as a function of time (Fig. 2). The percent of radioactivity recovered in extracted lipid fractions of 10^7 *P. marinus* cells incubated with 87 nM ^{3}H serine for 0.5, 1, 2 and 3 h were 3.3 ± 0.2, 5.2 ± 1.2, 5.6 ± 0.8 and 5.4 ± 0.8, respectively. Radioactivity was recovered in PS, PC, PE and LPC fractions. The lipid class with the highest incorporation rate of ^{3}H serine label was PS followed by PC, PE and LPC (Fig. 2).

Incubations of 90 nM ^{3}H myo-inositiol with 10^7 *P. marinus* cells resulted in recovery of radiolabel from the substrate in the PI fraction (Fig. 3). Incorporation increased from 0.5 h (1.2 ± 0.9 pmole) to 1 h (3.4 ± 1.2 pmole) but did not increase further at 2 h (2.6 ± 0.3 pmole) or 3 h (3.0 ± 0.2 pmole). Neutral lipids contained less than 5% of the incorporated radiolabel.

3.2. Concentration-dependence of serine incorporation

About 90 min incubations with 1, 10, 100 and 344 μM labeled serine resulted in recoveries in extracted *P. marinus* lipids of 2.11 ± 0.46, 1.84 ± 0.38, 1.35 ± 0.39 and 0.97 ± 0.08%, respectively. In 90 min incubations with labeled serine substrate concentrations of 1 and 10 μM the highest mean incorporation was into the PS fraction, followed by PC, PE and LPC, respectively (Table 1 and Fig. 4). The 100 μM serine incubation had the highest recovery of radiolabel in PS and PC fractions followed by PE and LPC, respectively (Table 1 and Fig. 4). At the highest serine concentration (344 μM) the most radioactivity was recovered in the PC fraction followed by PS, PE and LPC, respectively (Table 1 and Fig. 4).

Analysis of methyl esters of radiolabeled lipid fractions from incubations conducted with serine resulted in the detection of some label present in the fatty acid fractions. PC, PS and PE synthesized using ^{3}H serine were found to have less than 20% of the label in the fatty acid portion of the phospholipid, with over 80% in the

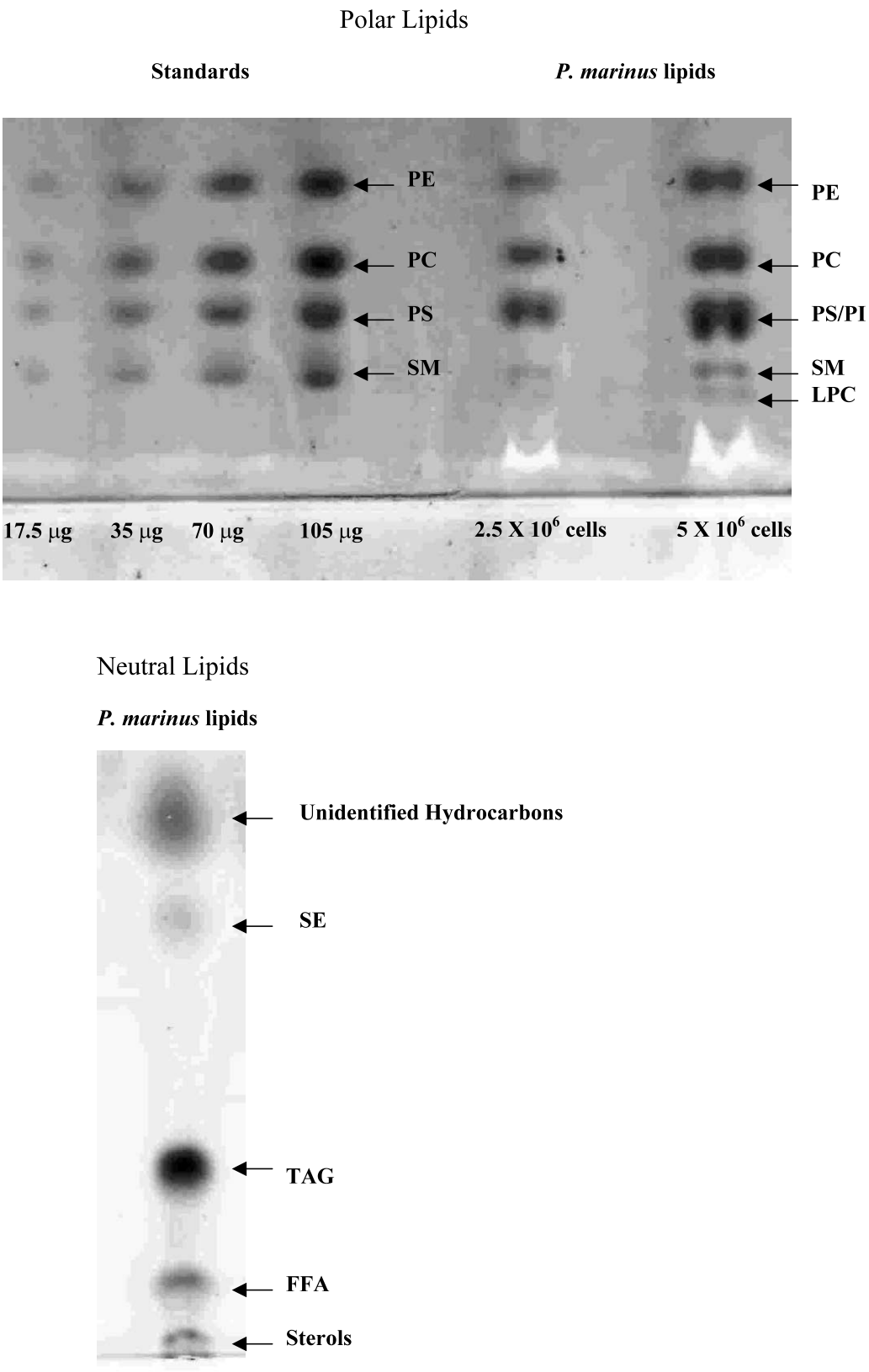

Fig. 1. Top: Separation of polar lipid standards and polar lipids from lipid extracts of *P. marinus* cells via HPTLC. Spots were visualized by spraying with 3% cupric acetate in 8% phosphoric acid and charred at 180 °C for 20 min. Mass values for standard lanes refer to the mass of each component in the standard mixture. Bottom, separation of neutral lipid classes from lipid extracts of *P. marinus* by HPTLC. Visualization of lipid classes was conducted as described above: Lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), Free Fatty acids (FFA), triacylglycerol (TAG), sterol esters (SE).

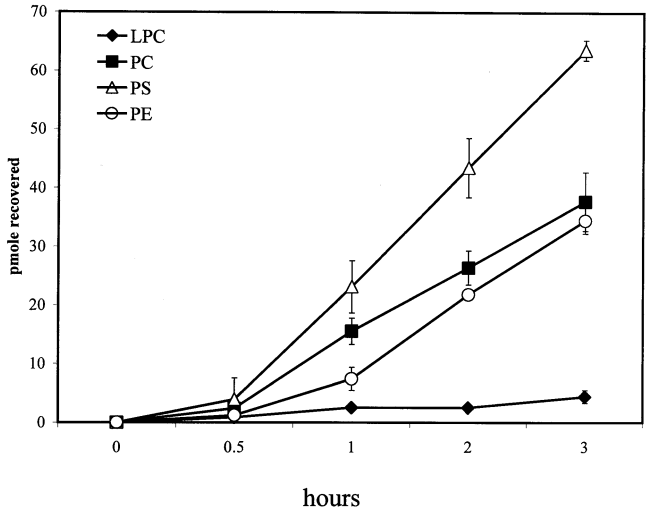

Fig. 2. Timecourse of recovery of ^{3}H serine in phospholipid fractions of *P. marinus*. Serine concentration was 87 nM using 10^{7} cells ($n=3$). Lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), FreeFatty acids (FFA), triacylglycerol (TAG), sterol esters (SE).

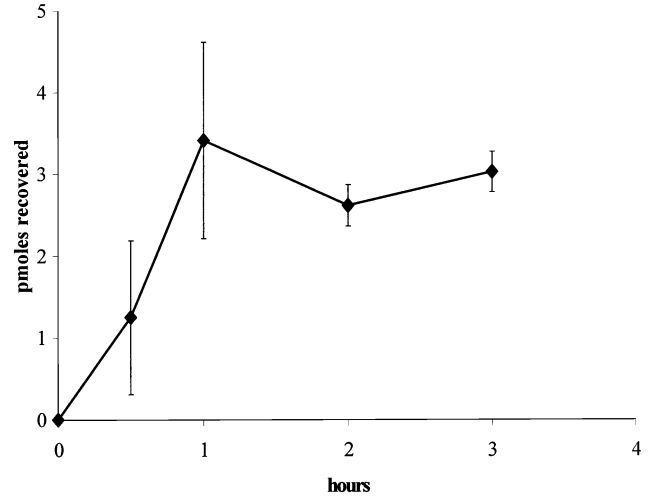

Fig. 3. Time–course for recovery of ^{3}H myo-inositol in the phosphatidylinositol fraction of *P. marinus* lipids. Incorporation was determined using 10^{7} cells incubated with 90 nM inositol ($n=3$).

polar headgroup/glycerol fraction. However, analysis of the total polar lipid fraction from serine incubations (including unidentified polar lipid components) and the neutral lipid fraction (including triacylglycerols, free fatty acids and sterol esters) revealed considerable incorporation in neutral lipids. While labeled lipids in the polar lipid fraction increased from 0.1 nmoles at 1 μM serine up to almost 2 nmoles at 344 μM serine in a 90 min incubation, neutral lipid incorporation increased from 0.1 nmoles to over 8 nmoles (Fig. 5).

3.3. Incorporation of choline and ethanolamine

When *P. marinus* cells were incubated with 344 μM ^{14}C choline and ^{14}C ethanolamine both resulted in the recovery of labeled phospholipids (Fig. 6). Of the total substrate added to the cultures, 0.82±0.19% of the choline and 2.04±1.21% of the ethanolamine were recovered in extracted lipids. Analyses of neutral lipid fractions from incubations using choline and ethanolamine revealed that the percentages of total incorporated label recovered in neutral lipids were less than 1 and 6%, respectively.

In the choline incubations, 89% of the label recovered in phospholipids was in the PC fraction (191.8±7.4 pmole). After 90 min incorporations of 10^{7} *P. marinus* cells with 344 μM ^{14}C choline the activities recovered in PC, LPC, PS and PE were, 191.9±7.4, 9.1±1.8, 13.6±8.1 and 1.5±2.6 pmole, respectively (Fig. 6). The incorporation rates of choline into each of these lipid classes were 238.9±9.2 pmole mg protein^{-1} h^{-1} for PC, 11.3±2.3 pmole mg protein^{-1} h^{-1} for LPC, 16.9±10.1 pmole mg protein^{-1} h^{-1} for PS and 1.9±3.2 pmole mg protein^{-1} h^{-1} for PE.

In the ethanolamine incubations, 49% of the label recovered in phospholipids was found in the PE fraction, with PS, LPC and PC containing 23, 14 and 14%, respectively. After 90 min incorporations of 10^{7} *P. marinus* cells with 344 μM ^{14}C ethanolamine the activities recovered in PE, PS, LPC and PC were 228.0±30.9, 101.9±8.6, 59.0±19.9 and 49.8±22.1 pmole, respectively (Fig. 6). These correspond to incorporation rates into PE, PS, LPC and PC of 283.9±38.5, 126.8±10.7, 73.4±24.8 and 62.0±27.5 pmole mg protein^{-1} h^{-1}, respectively.

Total incorporation of serine, ethanolamine and choline into combined phospholipid classes of 10^{7} *P. marinus* cells after 90 min were 712.7±67.8, 438.7±52.3 and 216.0±32.1 pmoles, respectively (Fig. 7). These values correspond to total incorporation rates of serine, ethanolamine and choline of 887.2±84.4, 546±65.1 and 268.9±21.4 pmole mg protein^{-1} h^{-1}, respectively. The incorporation rates for each of these substrates at 344 μM were significantly different (ANOVA, $P<0.01$).

4. Discussion

In eukaryotes there are at least two distinct pathways for the de novo synthesis of phospholipids. The first pathway is the so-called 'Kennedy pathway' (Weiss et al., 1958) in which the headgroup is first phosphorylated, then activated by the formation of a cytidine diphosphate (CDP) intermediate and finally attached to diacylglycerol (DAG) by a cytidylyltransferase to form the phospholipid. This direct pathway utilizes choline, and in some cases ethanolamine, as the headgroup to form PC and PE, respectively [29].

In the second phospholipid synthesis pathway, DAG is first activated by a cytidylyltransferase to form CDP-DAG. Two enzymes, PS synthase and PI synthase, can

Table 1
Incorporation of radiolabel from serine into phospholipid classes of *P. marinus*

Serine concentration (μM)	pmole mg protein^{-1} h^{-1} recovered in phospholipids				pmole 10^7 cells^{-1} recovered in phospholipids			
	PS	PE	PC	LPC	PS	PE	PC	LPC
1	11.0±4.6	5.9±2.3	7.4±1.4	0.6±0.1	9.5±3.7	4.7±1.8	5.9±1.1	0.5±0.1
10	138.7±19.1	58.4±12.5	59.2±13.6	5.1±1.6	70.5±15.3	46.9±10.0	47.5±10.9	4.1±1.3
100	226.4±33.3	185.1±28.7	225.7±46.5	39.3±11.4	181.9±26.7	148.7±23.1	181.3±37.4	31.5±9.1
344	232.1±18.2	171.3±31.2	417±30.1	66.8±13.1	186.4±14.6	137.6±25.1	335.0±24.2	53.6±10.5

Recovery of ^{3}H serine in phospholipid fractions of *P. marinus* at different substrate concentrations. Incubations were conducted for 90 min using 10^7 cells. All values are mean±S.D. ($n=3$). Lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), FreeFatty acids (FFA), triacylglycerol (TAG), sterol esters (SE).

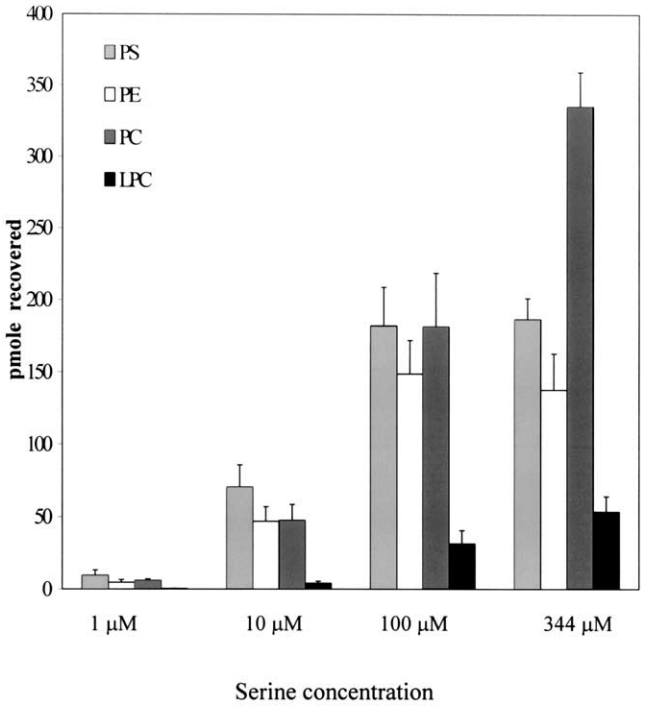

Fig. 4. Recovery of ^{3}H serine in phospholipid fractions of *P. marinus* at different substrate concentrations. Incubations were conducted for 90 min using 10^7 cells ($n=3$). Lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), FreeFatty acids (FFA), triacylglycerol (TAG), sterol esters (SE).

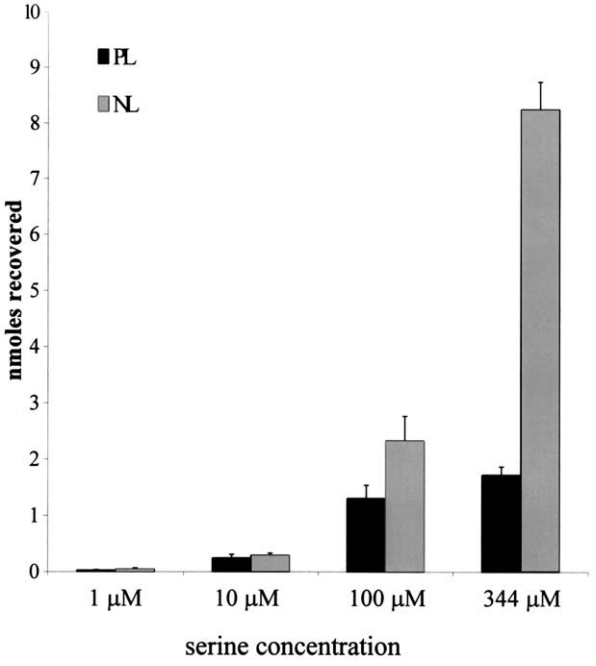

Fig. 5. Recovery of ^{3}H from serine in polar and neutral lipid fractions of *P. marinus*. Incubations with the substrate were for 90 min with 10^7 cells ($n=3$). NL, neutral lipids; PL, polar lipids.

then catalyze the formation of PS and PI from CDP-DAG and the polar headgroups serine and inositol, respectively. PS can be further metabolized by a reversible decarboxylation reaction to form PE which, in turn, can be converted to PC by phosphatidylethanolamine-*N*-methyltransferase [29]. PS can also be formed by headgroup exchange between PC or PE and serine and PE can be formed by decarboxylation of serine to ethanolamine followed by entry into the CDP-ethanolamine pathway [29].

Results from this study suggest that *P. marinus* is capable of utilizing both the Kennedy pathway and the CDP-DAG pathway for synthesizing phospholipids. The recovery of radiolabeled PC and PE from cells incubated with choline and ethanolamine, respectively, suggests that *P. marinus* utilizes both choline and ethanolamine as substrates for the Kennedy pathway. The recovery of radiolabled PS and PI from cells incubated with serine and inositol, respectively, suggests that *P. marinus* can utilize the CDP-DAG pathway for PS and PI synthesis. Recovered PE containing label originating in the serine substrate was probably made via decarboxylation of PS while labeled PC was synthesized from the PE via three rounds of methylation by the enzyme by phosphatidylethanolamine-*N*-methyltransferase. LPC is presumably a transient intermediate of PC.

The composition of polar lipids of *P. marinus* grown on the medium used in this study is 50% PC, 25% PE, 10% PS/PI, 10% cardiolipin and 5% sphingomyelin [24]. As such, the primary membrane component required for growth and proliferation is probably PC. Results from

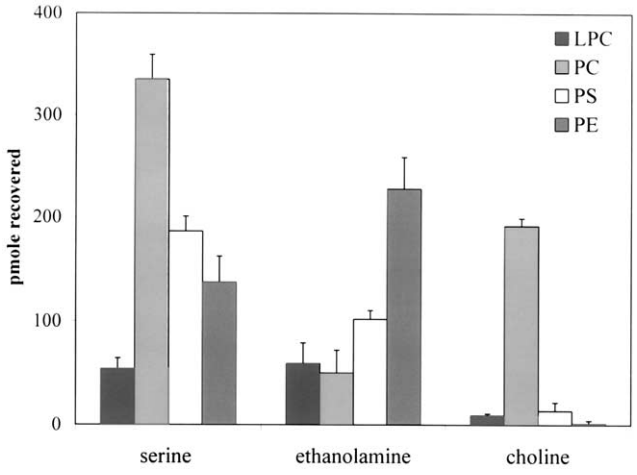

Fig. 6. Recovery of radiolabeled ethanolamine, choline and serine in phospholipid fractions of *P. marinus*. All substrates were 344 μM and incubations were conducted for 90 min with 10^7 cells ($n = 3$). Lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), FreeFatty acids (FFA), triacylglycerol (TAG), sterol esters (SE).

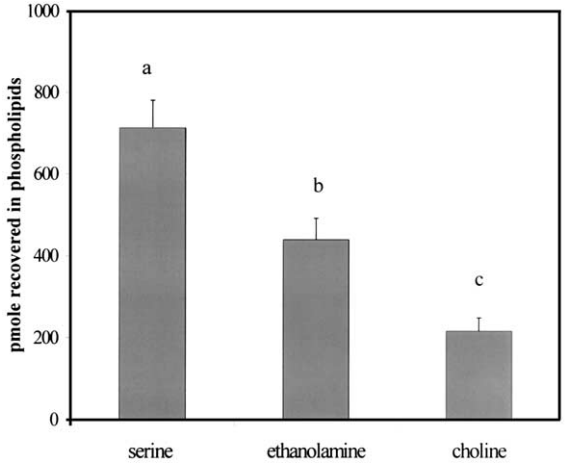

Fig. 7. Recovery of 344 μM ^{3}H serine, ^{14}C ethanolamine and ^{14}C choline in total phospholipids of 10^7 *P. marinus* cells after 90 min incubations ($n = 3$). Different letters denote values that are significantly different at the $P < 0.01$ level.

the present study along with previous work in our laboratory suggest that *P. marinus* is capable of obtaining PC by many different pathways: uptake of PC directly from the environment [21]; interconversion of environmentally derived PE to PC [24]; synthesis from choline via the CDP-choline pathway; synthesis from ethanolamine via CDP-ethanolamine pathway; synthesis from serine via the CDP-DAG pathway and possibly via recycling of LPC. While it is possible that some PS is formed via headgroup exchange with PC or PE, the combined activity in PE and PC from serine incubations is nearly twice that recovered in PS (Fig. 4). This strongly suggests that most of the observed activity is actual phospholipid biosynthesis and not just a rearrangement of headgroups in existing phospholipids. The observation that recovery of activity in the PS fraction is higher than the activity in the PE fraction in all serine incubations (Figs. 2 and 4) also suggests primary usage of the CDP-DAG pathway for the serine substrate. At the highest concentration of serine substrate tested (344 μM) the PC fraction contained the highest activity. This suggests that the CDP-DAG pathway is using serine to produce more PC (Fig. 4).

While a number of parasitic or pathogenic protozoans such as *G. lamblia* [26,30], *Trichomonas vaginalis*, *Toxoplasma gondii* [31] and *Pneumocystis carnii* [32] must rely on their host to provide them with pre-formed phospholipids, others, including the trypanosomids and *Plasmodium* species, have been shown to have the ability to synthesize a wide range of phospholipids from host-derived precursors. *Crithidia fasciculata* is known to synthesize PE and PC via the Kennedy pathway [33]. *Plasmodium* species also have been shown to utilize the CDP-choline pathway for the formation of PC, the CDP-ethanolamine pathway for the formation of PE and the CDP-DAG pathway for the synthesis of PI [25]. It is known that in the CDP-choline and CDP ethanolamine pathways the kinase reactions in *P. knowlesi* and *P. falciparum* are synthesized by two distinct enzymes, choline kinase and ethanolamine kinase [34]. The gene for a key enzyme in the CDP-choline pathway, CTP-phosphocholine cytidylyltranserferase, has been isolated from *P. faliciparum* and expressed in *Escherichia coli* [35]. While it is known that *Plasmodium* species can decarboxylate PS to PE [36] the use of the CDP-DAG pathway for the synthesis of PS is suspected, but not confirmed [25]. Trypanosomids have been shown to utilize the Kennedy pathways to make PC and PE and the CDP-DAG pathway to synthesize PI [31,37]. *Trypanosoma brucei*, like *Plasmodium* species, possesses separate sets of enzymes for synthesizing PC and PE via the Kennedy pathway [38]. Trypanosomids of the genus *Leishmania* also appear to have fully developed phospholipid synthetic capabilities [31]. The results presented here suggest that *P. marinus* is comparable in phospholipid synthetic capabilities to *Trypanosoma* and *Plasmodium* species.

Rates of phospholipid biosynthesis in *P. marinus* are comparable to those reported for *Plasmodium* species, although differences in methodology make direct comparisons difficult. Using *P. knowlesi*-infected erythrocytes, incubated with 320 μM ^{3}H choline, Vial et al. [39] reported PC synthesis rates of corresponding to approximately 80 pmole h^{-1} per 10^7 cells. In the present study, using ^{14}C choline at 344 μM a PC synthesis rate of 144 pmole h^{-1} per 10^7 *P. marinus* cells (Fig. 6) was recorded. Using 4–10 μM ^{14}C serine, Eabbadi et al. [36] reported incorporation rates into *P. knowlesi*-infected erythrocytes of 0.52 pmole h^{-1} per 10^7 cells for PS, 1.26

pmole h^{-1} per 10^7 cells for PE and 0.07 pmole h^{-1} per 10^7 cells for PC. In *P. marinus* incubated with 10 μM ^{3}H serine incorporation rates into PS, PE and PC of 46, 33 and 34 pmole h^{-1} per 10^7 cells, respectively, were found (Table 1, Fig. 4). These comparisons must be interpreted with caution due to the fact that incubations using *Plasmodium* species were conducted with infected erythrocytes, although the mature erythrocyte lacks any phospholipid biosynthesis capacity [31,40], whereas the experiments using *P. marinus* utilized axenic cell cultures.

While *P. marinus* cells did incorporate ^{3}H inositol into PI, activity levels were low compared with serine and the incorporation into PI did not increase after 1 h (Fig. 3). The lack of linearity past 1 h may be explained by the further metabolism of PI into other polar compounds. It is well known that parasitic protozoans utilize a glycosyl phosphatidylinositol (GPI) anchor to attach variant surface proteins to the cell surface [29]. Although GPIs have not yet been reported in *P. marinus*, it is possible that they are present in this organism. GPI anchors are major membrane components in other parasitic protozoans including the members of the genera *Plasmodium*, *Leishmania* and *Trypanosoma* and there is much interest in their potential as drug targets [41]. In this study, bioconversion of synthesized PI into other more polar compounds, such as GPIs or GPI precursors, may explain the lack of increase in PI after more than 1 h.

The finding of radiolabel from serine in neutral lipids and the fatty acid components of phospholipids is not surprising given the general utility of this molecule in both catabolic and anabolic pathways. We have previously reported that *P. marinus* can synthesize fatty acids from sodium acetate [23], so it is not unexpected that *P. marinus* may have utilized serine present in the media, probably via preliminary catabolism to acetyl CoA, as a substrate for fatty acid biosynthesis. It is noteworthy that this activity increases greatly at higher serine concentrations (Fig. 5). At serine concentrations of 1–10 μM the ratio of neutral to polar lipids containing radiolabel originating from serine is close to 1:1, whereas at 100 μM it is almost 2:1 and at 344 μM it is 4:1. It may be that at lower serine concentrations most of the available serine is required for phospholipid and protein synthesis, while at higher concentrations excess serine can be converted to FFA and glycerol then assembled into TAG for use as an energy store. If this is the case, such a strategy could be very useful to a parasite such as *P. marinus*. *P. marinus* has no known intermediary host and is known to infect oysters via dispersal through the water column [9,10]. The ability to sequester excess nutrients and store them as TAG may play a role in determining the viability and infectivity of dispersed cells.

The present study demonstrates that all major phospholipid classes, other than PI, can be synthesized by *P. marinus* using the amino acid serine as a substrate. The capability of *P. marinus* to use serine for synthesizing headgroups of polar lipids and the previously demonstrated ability to synthesize a wide range of saturated, mono-unsaturated and polyunsaturated fatty acids de novo using acetate [23], suggests that this parasite does not require preformed phospholipids from its host.

Pathways involved in the synthesis, modification or acquisition of phospholipids are especially promising drug targets in parasitic protozoans due to the requirement of new membrane for parasite replication [25]. Recently developed drugs with anti-protozoan properties that work through the disruption of lipid metabolism include Triclosan, which inhibits fatty acid synthesis in *P. falciparum* [42] and G25, an inhibitor of a choline transporter in erythrocytes infected with *P. falciparum* [43]. The effectiveness of these drugs against *P. marinus* has not yet been tested, however, the similarities in lipid metabolism of *P. falciparum* and *P. marinus* suggest that they may be effective against *P. marinus*.

In summary, *P. marinus* has a well developed capability to de novo synthesize all major phospholipids (PC, PE, PS and PI), requiring only an amino acid (serine) and sugar (inositol) as headgroup precursors. Due to the capability of maintaining *P. marinus* in axenic culture on defined media, it may make an excellent model system for studying the nutritional requirements of parasitic protozoans in relation to phospholipid biosynthesis and the host–parasite relationship in lipid metabolism.

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