Enzymes of phospholipid metabolism in the plasma membrane *of Acanthamoeba castellanii*

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Abstract Phospholipase A, lyophospholipase, acyl CoA hydrolase, and palmitoyl CoA synthetase are present in the plasma membrane of *Acanthamoeba castellanii*. The first three of these enzymes also occur in other cell fractions but in concentrations too low for the activities in the plasma membrane fraction to be accounted for by contamination by any other cell fraction. Palmitoyl CoA synthetase is restricted almost entirely to the plasma membrane and microsomal fractions; the microsomal activity is too low for the plasma membrane activity to be due to contamination by microsomes. Acyl CoA:lysolecithin acyltransferase is predominantly localized in the microsomal fraction, but the activity of the plasma membrane is probably too great to be accounted for by microsomal contamination. CDPcholine:1,2-diglyceride cholinephosphotransferase is restricted almost entirely to the microsomal fraction. Phospholipase C was not detected in any cell fraction or in the growth medium.

Supplementary key words phospholipase A_2 · lysophospholipase · phospholipase A_1 · acyl CoA synthetase · acyl CoA hydrolase · CDPcholine:1,2-diglyceride cholinephosphotransferase · acyl CoA:monoacylglycerophosphorylcholine · acyltransferase

Acanthamoeba castellanii is an organism particularly well suited for the study of dynamic plasma membrane phenomena. Its subcellular organization is similar to that of higher organisms, and it can be grown axenically under defined conditions and thus is amenable to bacterial culture methodology. Moreover, all pertinent available data (1, 2) suggest that *Acanthamoeba* does not actively transport solutes. As a result, it depends totally upon pinocytosis and phagocytosis for the uptake of nutrients. These endocytic processes critically involve the plasma membrane in events of membrane fusion and translation.

Our laboratory has recently reported the isolation and composition of *Acanthamoeba* plasma membranes (3, 4). The major phospholipids present are phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. This report is about those enzymes in the plasma membrane that may function in the turnover of plasma membrane phospholipids.

MATERIALS AND METHODS

Isolation of cell fractions

Amoeba plasma membrane and other subcellular fractions were isolated according to previously published procedures (3) and stored in pellet form at −20°C until needed. Rat liver microsomes were isolated by differential centrifugation of 10% (w/v) liver homogenates (5).

Labeled substrates

Labeled lysophosphatidylcholine was obtained by silicic acid column chromatography of labeled rat liver lipids. A halothane-anesthetized rat was injected intracardially with 1 ml of a 5% (w/v) solution of bovine serum albumin complexed to approximately 200 μCi of [1-^{14}C]palmitic acid. After 1 hr, the rat was killed and the liver lipids were extracted (6). The radioactive lysophosphatidylcholine would be predominantly, if not exclusively, 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine, because 1-acyllysophosphatidylcholine accounts for 80% of rat liver lysophosphatidylcholine (7), 2-acyllysophosphatides are unstable and readily isomerize to 1-acyllysophosphatides (8), and palmitic acid is preferentially incorporated into the 1-position of rat liver lecithin (9). The specific activity of the lysophosphatidylcholine was 6.7×10^4 cpm/μmole.

1-Acyl(saturated)-2-[1-^{14}C]linoleoyl-3-*sn*-phosphatidylethanolamine was prepared by acylating lysophosphatidylethanolamine in the presence of rat liver microsomes (10). A suspension of 0.5 mg of lysophosphatidylethanolamine and 0.24 mg, 50 μCi, of [1-^{14}C]linoleic acid in 0.5 ml of 0.125 M KCl–0.1 M Tris–HCl, pH 7.2, was combined with 0.5 ml of buffered 1 mM CoASH–50 mM ATP, pH 7.2, and sonicated for 1 min. An additional 0.5 ml of CoA–ATP solution was then added, followed by 2 ml (40 mg of protein) of liver microsomal suspension. After incubating for 60 min at 37°C, the reaction volume was doubled by adding water, and the lipids were then extracted. Pure phosphatidylethanolamine was isolated by

preparative thin-layer chromatography on 0.5-mm silicic acid plates in chloroform–methanol–water 65:35:4 (by vol). Its specific activity was 9×10^5 cpm/μmole. An analysis of the hydrolysis products after snake venom phospholipase A_2 action showed that over 98% of the label was located at the 2-position of the phospholipid molecule. 1-Acyl(saturated)-2-[1-^{14}C]oleoyl-3-*sn*-phosphatidylcholine was similarly synthesized from lysophosphatidylcholine and [1-^{14}C]oleic acid. Its specific activity was 2.3×10^6 cpm/μmole, with over 99% of the label being at the 2-position.

[1-^{14}C]Oleoyl CoA was synthesized by the procedure of Goldman and Vagelos (11) and purified according to either their method or that of Pullman (12). The compound was characterized by thiol ester determination (13), phosphate analysis (14), ultraviolet absorption ratios, and comparison of its ultraviolet absorption spectrum with that of commercial oleoyl CoA obtained from P-L Biochemicals, Milwaukee, Wis. The following absorption ratios were obtained: 250:260 nm = 0.90, 280:260 nm = 0.14, and 232:260 = 0.58. Based on all the criteria, the purity of the [1-^{14}C]oleoyl CoA was judged to be greater than 90%. Its specific activity was 3.5×10^6 cpm/μmole.

Enzyme assays

Unless otherwise indicated, enzymatic activities for all subcellular fractions were determined under conditions in which the rates of reaction were linearly dependent on the protein concentration and were constant during the period of incubation.

The standard assay for phospholipase A activity contained 100 μM ^{14}C-labeled phospholipid, 10 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.5, and 100 μg of protein in a final volume of 1.0 ml. Phospholipid dispersions at 1 mM were achieved by sonication for 1 min with a microprobe or by placing a vial containing the suspension in a sonicating cleaning bath for 10 min. The mixture was incubated at 37°C, and the reaction was terminated after 15 min by the addition of 0.1 ml of 1 N H_2SO_4 followed by lipid extraction. Thin-layer chromatography was carried out on 5 × 20 cm plates, and, after a brief exposure to iodine vapors, the areas corresponding to fatty acid, phospholipid, and lysophospholipid were scraped directly into scintillation vials containing 10 ml of Aquasol (New England Nuclear, Boston, Mass.) or 15 ml of a mixture previously described (5).

Phospholipase A activity with endogenous substrate was assayed with plasma membranes obtained from cells grown in the presence of [1,2-^{14}C]ethanolamine · HCl. A 1-l culture of amoebae was inoculated in the log phase with 200 μCi of ^{14}C-labeled ethanolamine 24 hr before harvesting the cells from which plasma membranes were isolated. Plasma membranes, 125 μg of protein, were incubated in 0.5 ml of 50 mM Tris–HCl, pH 7.2, containing 2 mM $CaCl_2$, 0.5 mM EGTA, and 66 mM KCl. Aliquots of 0.1 ml were removed at timed intervals, and extracted by the method of Bligh and Dyer (15); the lipid was separated by thin-layer chromatography and counted.

Acyl CoA:monoacylglycerophosphorylcholine acyltransferase was assayed spectrophotometrically by measuring the release of CoASH with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at 413 nm (16). Amoeba plasma membranes exhibit both acyltransferase and acylhydrolase activities. Therefore, the acyltransferase values were calculated from the increase in rate in the presence of acyl acceptor, monoacylglycerophosphorylcholine, over that measured in its absence (acylhydrolase rate). The standard assay contained, in a final volume of 1 ml, 1 mM DTNB, 50 mM Tris–HCl, pH 7.5, 50 μM acyl CoA, 150 μM monoacylglycerophosphorylcholine (when measuring acyltransferase), and different amounts of enzyme protein. Acyltransferase was also assayed radiochemically with 1-^{14}C-labeled acyl CoA as substrate in an incubation mixture similar to that used in the spectrophotometric assay but without DTNB. Upon completion of the reaction, the lipids were extracted (15) and separated chromatographically. The areas corresponding to phosphatidylcholine and fatty acid were scraped off the plate and counted directly by liquid scintillation spectrometry.

Lysophospholipase activity was determined by measuring the fatty acid released from 1-[1-^{14}C]palmitoyllysophosphatidylcholine after incubation with subcellular fractions (17). Each membrane fraction incubation mixture contained 50 mM Tris–HCl, pH 7.5, 250 μM [^{14}C]lysophosphatidylcholine, and 10 μg/ml membrane protein in a final volume of 2 ml. The reaction was carried out at 37°C for 10 min. Under these conditions, the rate of reaction was directly proportional to the concentration of plasma membrane protein, but the soluble phase lysophospholipase was strongly inhibited. The latter activity was measured in a mixture containing 50 mM Tris–HCl, pH 7.5, 100 μM [^{14}C]lysophosphatidylcholine, and 1 mg/ml protein in a final volume of 2 ml.

Palmitoyl CoA synthetase was assayed in a reaction mixture that contained 0.1 M Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM CoASH, 10 mM ATP, 50 μM [1-^{14}C]palmitate-K^+ salt, and enzyme protein in a final volume of 0.2 ml. The incubation was carried out at 37°C and was terminated after 5 min by extraction of fatty acids according to Dole and Meinertz (18). After washing the aqueous layer four times with heptane, aliquots of the aqueous layer were removed, mixed with Aquasol, and counted by liquid scintillation spectrometry.

The standard assay for CDPcholine:1,2-diacylglycerol cholinephosphotransferase contained 0.06% Tween 20, 100 mM Tris–HCl, pH 7.5, 5 mM reduced glutathione,

TABLE 1. Subcellular distribution of enzymes of phospholipid metabolism in *Acanthamoeba castellanii*[a]

Enzyme	Plasma Membrane	Total Homogenate	Mitochondria	Microsomes	Soluble Phase
Phospholipase A					
Radioactive fatty acid[b]	5.0	8.0	13.5	2.7	6.9
Radioactive lysophospholipid[b]	1.6	2.6	11.4	4.8	1.9
Oleoyl CoA lysolecithin acyltransferase	2.4	3.4	0.1	10.4	0.4
Oleoyl CoA hydrolase	1.6	2.1	2.2	4.8	0.7
Lysophospholipase	8.8	3.6	4.1	6.6	6.9
Palmitoyl CoA synthetase	8.2	4.4	1.5	19.8	1.0
CDPcholine:1,2-diacylglycerol cholinephosphotransferase	0.024	0.041	0.021	0.361	

[a] Specific activities in nmoles/min/mg of protein. The plasma membrane values are the averages of four to nine determinations.

[b] Substrate was 2-[^{14}C]linoleoyl-3-*sn*-phosphatidylethanolamine. Radioactive lysophospholipid must be the product of phospholipase A_1. As discussed more fully in the text, radioactive fatty acid could be formed by phospholipase A_2 or by the sequential action of phospholipase A_1 and lysophospholipase.

100 μM CDPcholine (9.0×10^5 cpm/μmole), 10 mM 1,2-diglyceride, and enzyme protein in a final volume of 0.5 ml (19). Incubation was carried out for 15 min at 37°C. At the end of the reaction, lipids were extracted (14, 19) and aliquots of the chloroform phase, which contained labeled phosphatidylcholine only, were counted.

Phospholipase C activity was assayed in a medium containing 10 mM $CaCl_2$, 0.01 M Tris–HCl, pH 7.5, and protein in a total volume of 0.5 ml. To this mixture was added 2.0 ml of ether containing 0.5 mM [^{14}C]ethanolamine-labeled phospholipid (2×10^4 cpm/nmole, isolated from amoebae grown in the presence of [1,2-^{14}C]ethanolamine). The reaction mixture was agitated continuously on a vortex mixer. The release of radioactive phosphorylethanolamine into the aqueous phase was measured in aliquots that were added directly to Aquasol and then counted by liquid scintillation spectrometry. The incubation continued for as long as 30 min with as much as 0.5 mg of protein. In addition to assaying the membrane fractions, possible extracellular production of phospholipase C was also monitored by assaying the cell culture medium. Cells were removed by centrifugation, and amoeba-free culture medium was dialyzed for 24 hr against 0.01 M Tris–HCl, pH 7.4. The dialyzed medium was centrifuged at 105,000 *g* for 90 min, and the supernatant solution was saved for enzymatic determinations. Analysis of the dialyzed medium indicated that a significant amount of protein was present. This may have been protein secreted into the medium by *Acanthamoeba* or released by cell breakage. No phospholipase C activity was detected in any fraction under conditions that showed extensive activity for a crude commercial preparation of phospholipase C from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, Mo.).

Chemical determinations

Protein concentrations were determined according to Lowry et al. (20), with bovine serum albumin as the standard. Inorganic phosphate determinations were carried out by the method of Chen, Toribara, and Warner (14). Acyl ester groups were assayed as described by Snyder and Stephens (13), with methyl stearate as the standard.

Commercial products

1-^{14}C-labeled fatty acids, stearoyl CoA and palmitoyl CoA, CDP-[^{14}C]choline, and [1,2-^{14}C]-ethanolamine were obtained from New England Nuclear. Unlabeled CoA esters were purchased from P-L Biochemicals. Reference lipids were products of Serdary Biochemicals, London, Ontario, and Supelco, Bellefonte, Pa. Lyophilized snake venom was obtained from Sigma Chemical Co.

RESULTS

Phospholipase A activities

The data in **Table 1** indicate that phospholipase A activity is present in all the main cell fractions of *Acanthamoeba,* including the soluble phase. When 1-acyl(saturated)-2-[^{14}C]linoleoyl-3-*sn*-phosphatidylethanolamine was incubated with plasma membranes, 86% of the labeled reaction product was fatty acid and only 14% was labeled lysophosphatidylethanolamine. Since the substrate was labeled in the 2-position, radioactive lysophosphatidylethanolamine can only be the product of phospholipase A_1 activity. Radioactive fatty acid, however, could have been formed either by phospholipase A_2 acting directly on the phospholipid substrate or by the sequential action of phospholipase A_1 and lysophospholipase. We believe it likely that the plasma membranes contain a phospholipase A_2, in addition to phospholipase A_1, because the lysophospholipase activity of the plasma membrane seems to be insufficient to account for the observed rate of formation of radioactive fatty acids from the labeled phosphatidylethanolamine. In the 15-min incubation period, 24 nmoles of phosphatidylethanolamine was hydrolyzed (by measurement of residual substrate). If 24 nmoles of lysophosphatidylethanolamine had been present at the beginning of the

TABLE 2. Kinetic data for plasma membrane enzymes

Enzyme	Apparent V_{max}	Apparent K_m
	nmoles/min/mg protein	*M*
Phospholipase A[a]	9.8	2.7×10^{-4}
Oleoyl CoA lysolecithin acyltransferase	6.7	1.1×10^{-4}
Oleoyl CoA hydrolase	6.5	1.8×10^{-4}
Lysophospholipase	31	3.5×10^{-4}
Palmitoyl CoA synthetase	29	1.1×10^{-4}

[a] Rate of formation of radioactive fatty acid from 2-[^{14}C]linoleoyl-phosphatidylethanolamine.

incubation, we calculate from the reciprocal plot from which the apparent V_{max} and apparent K_m of the lysophospholipase were obtained (**Table 2**) that the rate of release of radioactive fatty acids would have been about 4 nmoles/min/mg of protein. Since the actual concentration of lysophosphatidylethanolamine was very much less than 24 nmoles/ml at all times during the incubation (the maximum concentration was 2.4 nmoles/ml), the activity of lysophospholipase should have been too low to account for the observed rate of formation of radioactive fatty acids (5 nmoles/min/mg of protein). Nonetheless, these data do not establish unequivocally the presence of phospholipase A_2 in the plasma membranes.

The apparent V_{max} and apparent K_m of the plasma membrane phospholipase activity are shown in Table 2. Although Michaelis-Menten kinetics are not strictly applicable to reactions in which the substrates are above the critical micelle concentration (and in reactions in which more than one enzyme may be involved), linear reciprocal plots were obtained for all of the reactions listed in Table 2, and the apparent Km and $Vmax$ values are useful empirical numbers.

The plasma membrane phospholipase shows almost no Ca^{2+} dependence although it is inhibited by other cations tested (**Table 3**). Detergents had an inhibitory effect (Table 3). **Fig. 1** shows that the plasma membrane enzyme is capable of acting on endogenous substrate that is presumably in a physical state closely resembling the situation in vivo. Exogenous phosphatidylcholine was hydrolyzed at less than 10% of the rate of exogenous phosphatidylethanolamine.

TABLE 3. Properties of plasma membrane phospholipase A

Reaction Conditions	Phosphatidylethanolamine Hydrolysis
	%
Control	100
No $CaCl_2$	89.5
EGTA, 50 mM	64.8
$ZnCl_2$, 10 mM	10.9
$HgCl_2$, 10 mM	51.7
Glutathione-SH, 10 mM	25.2
Deoxycholate, 0.05%	71.7
Sodium dodecyl sulfate, 0.05%	35.7

Conditions are the same as for the standard assay except as noted.

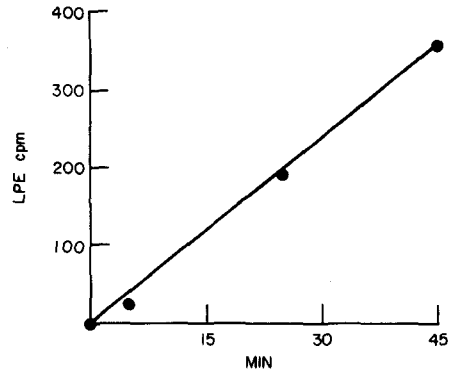

Fig. 1. Hydrolysis of endogenous phospholipid by *Acanthamoeba* plasma membrane phospholipase A assayed by the formation of radioactive lysophosphatidylethanolamine (LPE) from endogenous labeled phosphatidylethanolamine in plasma membranes isolated from cells grown in the presence of [^{14}C]ethanolamine. Experimental conditions are described in detail in the text. From the specific activity of the phosphatidylethanolamine, it can be calculated that the activity towards endogenous substrate is very nearly the same as towards exogenous substrate.

Acyl CoA:lysolecithin acyltransferase activity

Acyltransferase activity is primarily localized in the microsomal fraction, with significant activity also exhibited by the plasma membrane (Tables 1 and 2). The highest rates for the plasma membrane enzyme were obtained with oleoyl CoA, stearoyl CoA, and myristoyl CoA (**Table 4**). Oleic and stearic acids are among the most prevalent fatty acids present in the plasma membrane (3). In a series of separate experiments (not reported) it was demonstrated that triglyceride cannot serve as an acyl donor to the acyltransferase enzyme complex.

Acyl CoA hydrolase activity

The subcellular distribution of oleoyl CoA hydrolase is shown in Tables 1 and 2. All fractions have measurable activity, the microsomal fraction being the greatest, but the activity of the plasma membrane fraction (Tables 1 and 2) is too high to be accounted for by contamination by

TABLE 4. Specificity of the acyl CoA:lysolecithin acyltransferase of *Acanthamoeba* plasma membranes

Substrate	Activity
	nmoles/min/mg protein
Oleoyl CoA	2.4
Stearoyl CoA	2.7
Palmitoyl CoA	1.9
Myristoyl CoA	2.6
Lauroyl CoA	1.3
[^{14}C]Palmitoyl CoA	1.4
[^{14}C]Oleoyl CoA	2.8

Activity for plasma membranes measured as described for the standard assay.

any other fraction. The possibility of some of the activity being due to nonspecific esterases has not been ruled out.

Lysophospholipase

As indicated by the data in Table 1, both the soluble phase and plasma membrane fractions, in addition to other fractions, have a high lysophospholipase activity. The incubation conditions necessary for the activity to be proportional to enzyme concentration were very different for the soluble phase and plasma membrane fractions (Materials and Methods). The apparent K_m of 3.5×10^{-4} M calculated for the membrane-bound activity (Table 2) was approximately five times that found for the soluble enzyme. Although the membranous state of the enzyme might be sufficient to alter the behavior of a common enzyme, the possibility of the existence of two different enzymes cannot be excluded at this time.

Palmitoyl CoA synthetase

The results presented in Tables 1 and 2 indicate that palmitoyl CoA synthetase activity occurs primarily in the plasma membrane and microsomal fractions of *Acanthamoeba*.

CDPcholine:1,2-diglyceride cholinephosphotransferase

The localization of this activity is primarily in the microsomal fraction (Table 1). *Acanthamoeba* plasma membranes probably lack the enzymatic mechanism required for de novo phospholipid synthesis by the cytidine dinucleotide pathway (21). As mentioned before, this enzyme serves as a useful measure of the maximum possible contamination of the plasma membrane fraction by the microsomal fraction.

DISCUSSION

This laboratory has previously presented chemical, enzymatic, and ultrastructural evidence for the high purity of *Acanthamoeba* plasma membranes isolated by the method used in the present investigation (3). The enzymatic data presented in this paper offer additional evidence that neither microsomal nor mitochondrial contamination can account for most of the enzymes of phospholipid metabolism found in the plasma membrane. Thus, for example, assuming that plasma membranes contain no choline phosphotransferase activity, the maximal contamination by microsomes would be less than 10%. Similarly, mitochrondrial contamination cannot contribute significantly to the plasma membrane activities; there are several enzymes that have a higher specific activity in the plasma membrane fraction than in the mitochondrial fraction. However, it should be noted that possible contamination of the plasma membranes with other particulate fractions has not been quantitatively evaluated (e.g., Golgi apparatus and contractile vacuole). With the use of electron microscopic cytochemistry it has recently been shown (22) that, as previously suspected (3), quantitatively minor contamination by contractile vacuoles probably accounts for the alkaline phosphatase and 5′-nucleotidase activities of isolated *Acanthamoeba* plasma membranes. In the absence of suitable cytochemical techniques (attempts to apply the cytochemical assay of acyl CoA transferase [23] to *Acanthamoeba* have been unsuccessful thus far) for the enzymes of phospholipid metabolism, it is difficult to eliminate the possibility that one or more of the enzymatic activities found in isolated plasma membranes might be due to a minor contaminant with high specific activity. With this possible qualification, the enzymes reported in this paper are the only ones now known to be present in the plasma membrane of *Acanthamoeba castellanii*.

To our knowledge, no other plasma membrane from a eukaryotic cell has been found to contain as many enzymes involved in phospholipid metabolism as that of *Acanthamoeba*. Acyl CoA:lysolecithin acyltransferase (23–25; but see also 26, 27), lysophospholipase (5), and acyl CoA synthetase activities (28, 29; but see 30) are not present in plasma membranes from rat liver. Rat liver plasma membranes do contain phospholipases A_1 (31) and A_2 (31, 32) and acyl CoA hydrolase (24). Erythrocyte plasma membranes contain acyltransferase (33) and lysophospholipase (34), but phospholipase A activity is very low and is detectable only after trypsinolysis (33).

The *Acanthamoeba* cell surface is a highly specialized area involved directly in the uptake of nutrients by endocytosis, membrane fusion events, and cell motility. This laboratory has previously reported (2) that pinocytosis in *Acanthamoeba* is continuous and that internalization of the cell surface may result in plasma membrane turning over as much as 10 to 50 times per hour. This is much faster than the rates estimated for other cells (2). Lucy (35) has proposed that membrane fusion might be a consequence of controlled, local degradation of phospholipids by phospholipase A_2, leading to an instability of the membrane phospholipid bilayer, which would then re-form after the acylation of the lysophospholipid. All of the enzymes needed for such a cycle (phospholipase A_2, acyl CoA synthetase, and acyl CoA:lysolecithin transferase) are present in the *Acanthamoeba* plasma membrane. However, direct attempts to demonstrate stimulation of such a cycle by induced phagocytosis of latex beads were unsuccessful (36), perhaps because of the then-unknown high background level of continuing pinocytosis discussed above. More recent attempts[1] have also failed to demonstrate that the phospholipids of *Acanthamoeba* plasma membranes turn over more rapidly than the phospholipids of other cell membranes.

[1] Simmons, S., and E. D. Korn. Unpublished results.

The apparent absence of cholinephosphotransferase (Table 1) indicates that *Acanthamoeba* plasma membranes are incapable of synthesizing lecithin de novo by the cytidine dinucleotide pathway. As in rat liver (37), synthesis of lecithin appears to be confined almost entirely to the microsomal fraction of *Acanthamoeba,* in contrast to another protozoan, *Tetrahymena,* in which mitochondria have been reported to be the main site of lecithin synthesis (38). The plasma membrane of *Acanthamoeba* does, however, have the enzymatic capability of modulating the fatty acyl composition of phospholipids by deacylation and acylation (39).

The plasma membrane lysophospholipase may serve to protect the cell from the lytic effect of lysophospholipids either of exogenous origin or formed endogenously in digestive vacuoles, the membrane of which is derived from and in equilibrium with the plasma membrane. Finally, the acyl CoA synthetase and acyltransferase activities of the plasma membrane might be involved in the uptake of long-chain fatty acids previously shown to occur in *Acanthamoeba* (40). If so, the reaction catalyzed by acyltransferase would be the rate-limiting step, as in mammalian mitochondria (41).

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