Sterol effects on phospholipid biosynthesis in the yeast strain GL7

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Abstract Cells of the yeast sterol auxotroph GL7 were grown on either ergosterol or cholesterol to mid-logarithmic phase and total membrane fractions prepared. Activities of phospholipid biosynthetic enzymes in the two cell types were determined. The rates of phosphatidyl-ethanolamine-phosphatidyl-choline-N-methyl transferase and acyl-CoA-α-glycerol-3-phosphate transcylase were significantly greater in ergosterol-grown than in cholesterol-grown cells. These reactions were also inhibited by the polyene antibiotic filipin. By contrast the activities of long-chain fatty acyl-CoA synthetase, CTP-phosphatidate-cytidyl transferase, phosphatidylserine decarboxylase and of phosphatidylinositol synthetase were identical in the two (ergosterol and cholesterol) cultures and unaffected by filipin. The ergosterol effect on phosphatidyl-ethanolamine N-methyl transferase was greatest in cells harvested in early log phase, intermediate in the mid-log phase cells, and not significant in stationary phase cells. **—Ramgopal, M., M. Zundel, and K. Bloch.** Sterol effects on phospholipid biosynthesis in the yeast strain GL7. *J. Lipid Res.* 1990. **31:** 653–658.

Supplementary key words filipin • phosphatidyl-ethanolamine • cholesterol • N-methyl transferase • ergosterol

Recent reports from this laboratory provide evidence for dual and probably multiple roles of the sterol molecule per se in unicellular microorganisms. We designate these roles which are novel as metabolic. They are thought, at least in part, to account for the phenomenon of "sterol synergism," discovered when the growth responses of sterol auxotrophs to various structurally related sterols and their combinations were examined (1, 2). The principal outcome of these studies was to implicate the sterol molecule in the regulation of some selected reactions of phospholipid biosynthesis in the yeast strain GL7 (3–6). In parallel, independent investigations, Dahl, Biemann, and Dahl (7) have described another novel role for sterol, a marked stimulation of a membrane-associated GL7 protein kinase by ergosterol in concentrations as low as 10^{-8} M.

Here we present some further results obtained with the yeast auxotroph GL7, showing that the rates of two component reactions of phospholipid biosynthesis respond to sterol while four others do not.

MATERIALS AND METHODS

New England Nuclear supplied the following radiochemicals: [5-^{3}H]cytidine triphosphate, [1-^{14}C]oleic acid, S-adenosyl[methyl-^{3}H]methionine, [methyl-^{3}H]methionine, [1-^{14}C]oleoyl-CoA, [^{14}C]glycerophosphate, and [2-^{3}H]myoinositol; phosphatidyl-L-[U-^{14}C]serine [1,2 dioleoyl] was from Amersham Corp. Ergosterol, cholesterol, filipin, and oleic acid were from Sigma. The sterols were recrystallized from ethanol and acetone. Before use, filipin was recrystallized from chloroform–methanol, dissolved in 20 mM DMSO and the solution was heated to 50°C for 2 h.

Growth of *Saccharomyces cerevisiae* strain GL7

Cells of the sterol auxotroph GL7 (erg. 12, heme, 3, Gollub et al. (8)) were stored on slants and transferred to liquid medium containing 1 μg/ml of either cholesterol or ergosterol and 50 μg/ml of oleic acid as described (6). Cell growth was in air in the dark at 30°C and monitored by measuring absorbances at 540 nm. Unless otherwise stated, cells were harvested in mid-logarithmic phase (A_{540} = 0.4). Hemocytometer counting after staining with methylene blue showed that cell viability was more than 97% at the time of harvest.

Preparation of membrane fractions

Cultures of GL7 cells grown to an A_{540} of 0.4–0.5 (unless stated otherwise) as described above were

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; DDT, dithiothreitol; TLC, thin-layer chromatography.

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harvested, washed twice with Tris buffer (pH 7.4) containing 1 mM EDTA (buffer A). The cells were resuspended in the same buffer, disrupted by vortexing with 0.45–0.50 mm glass beads and centrifuged at 2300 *g* for 10 min. The supernatant was decanted and centrifuged at 100,000 *g* for 1 h. The resulting pellet was washed once and resuspended to a protein concentration of 10–20 mg/ml. These membrane suspensions were stored at −70°C until use. Protein concentrations were determined according to Lowry et al. (9).

Enzyme assays

Long-chain acyl-CoA synthetase. Assay mixtures, essentially as described by Tanaka, Hosaka, and Numa (10) contained 125 mM Tris, pH 7.5, 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 15 mM ATP (pH 7.1), 2.5 mM egg yolk PC, 1 mM CoA, 0.1% Triton X-100, and 0.2 mg/ml membrane protein in a final volume of 0.4 ml. After a 10-min preincubation, reactions were started by addition of [1-^{14}C]oleic acid (0.375 Ci/mol, final concentration 1 mM) and run for 10 min at 30°C. Reactions were terminated by addition of 1 ml of 1-propanol-n-heptane-1 M H_2SO_4 40:10:1 (v/v). Thereafter 2 ml of n-heptane and 1 ml of water added and the organic layer was discarded. The aqueous layer was washed four times with 2 ml of n-heptane containing 0.1 mg nonisotopic oleic acid/ml. The last wash contained no significant radioactivity. The aqueous phase containing the acyl CoA was then counted.

CTP-phosphatidic acid cytidyltransferase. The assay procedure was essentially that the Sparrow and Raetz (11). Solutions contained 60 mM phosphate buffer, pH 6.4, 2 mM phosphatidic acid, 1 mM [^{3}H]CTP (2.3 Ci/mol), 20 mM $MgCl_2$, 0.6–0.7 mg/ml of membrane protein in a final volume of 0.4 ml. The $MgCl_2$ was added to start reactions which were run for 15 min at 30°C. Addition of 1 ml of 0.1 M HCl in methanol terminated the reaction. After the addition of 50 μg of PC, 2 ml of chloroform, and 3 ml of 2 M KCl, the organic layer was washed three times with 3 ml each of 2 M KCl, evaporated to dryness, and the residue was dissolved in chloroform and applied to silica gel plates for TLC analysis. The solvent was chloroform–methanol–water–acetic acid 50:28:8:4. The band coinciding with standard CDP-diglyceride was transferred to vials for counting.

Phosphatidylserine decarboxylase. The assay mixture modified from that described by Carson et al. (12) contained 100 mM Tris, pH 7, 10 mm DTT, 10 mM EDTA, Triton X-100, 0.1%, 0.2 mM dioleoyl-*sn*-glycero-3-phospho-L-[U-^{14}C]serine (22 Ci/mol) and 0.5 mg of membrane protein. The reaction was started by addition of radioactive substrate and was run for 10 min at 30°C. Reactions were terminated by addition of 3 ml chloroform–methanol 2:1 (v/v) and 40 μg phosphatidylethanolamine (PE) as carrier. After addition of 0.8 ml 0.9% NaCl, the aqueous layer was discarded. The organic phase was washed with 2 ml of chloroform–methanol–0.9% NaCl 3:48:47, evaporated under nitrogen, and the residue was dissolved in chloroform–methanol 2:1 (v/v) and applied to silica gel plates for TLC analysis. Bands corresponding to authentic PC and PE were transferred to vials for counting. Radioactivity in PE was taken as a measure of PS decarboxylase activity. The solvent system was chloroform methanol water 65:25:4.

Glycerol-3-phosphate-acyl-CoA acyl transferase. Assays were performed according to the method of Schlossmann and Bell (13). Incubation mixtures contained 75 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 2 mg of fatty acid-free bovine serum albumin per ml, 8 mM sodium fluoride, 1 mM DTT, 80 μM palmitoyl-CoA or oleyl CoA and 0.9 mM [^{14}C]glycerol-phosphate (8.3 μCi/μmol) in a total volume of 0.6 ml. After a 5-min preincubation at 30°C, reactions were started by addition of 0.2 mg/ml of membrane protein. Cells harvested at an absorbance of 0.2–0.3 were the source of enzyme. At 10 min, 100-μl aliquots were removed and reactions were terminated by addition of 3 ml of chlorform–methanol 1:2 (v/v) and 0.7 mol of 1% perchloric acid per 100 μl of assay mixture. After further addition of 1 ml of chloroform and 1 ml of 1% perchloric acid, the aqueous phase was separated and discarded. The organic layer was washed three times with 2 ml of 1% perchloric acid, evaporated to dryness, and the residue was dissolved in chloroform and applied to silica gel plates for TLC analysis. The developing solvent was chloroform–methanol–water 64:27:4. The bands corresponding to lysophosphatidic acid (R_f = 0.24) and phosphatidic acid (R_f = 0.49) were transferred to vials and counted.

Phosphatidylinositol synthetase. Following the procedure of Fischl et al. (14) assay solutions contained 50 mM Tris buffer, pH 8.0, 2.5 mM $MnCl_2$, 0.5 mM myo[2-^{3}H]inositol (6,000 dpm/nmol), 0.2 mM CDP-diglyceride, and 0.5 mM Triton X-100 in a final volume of 0.6 ml. After 5 min of preincubation at 30°C, 1.2 mg of membrane protein was added to start the reaction and incubation was continued for 20 min, when 1.5 ml of water was added to terminate reactions. The organic phase was washed twice with methanol–water 1:3, evaporated to dryness, and the residue was applied to silica gel plates for TLC analysis. The band coinciding with authenic PI was transferred to vials for counting. The TLC solvent was chloroform–methanol–water–acetone–acetic acid 50:10:5:20:10. Only summary data are shown in Fig. 1.

Phosphatidylethanolamine-phosphatidylcholine-N-methyl transferase was assayed as previously described (6).

Effects of the polyene antibiotic filipin

Filipin recrystallized from chloroform–methanol was dissolved in DMSO to a final concentration of 2.5% and heated to 50°C for 2 h prior to use as described (6). For enzyme inhibition studies, assay mixtures were prein-

cubated with filipin for 10 min at 30°C before reactions were started.

RESULTS AND DISCUSSION

We describe here continuing studies on the regulation of phospholipid biosynthesis by sterols. In the past we have used two approaches to this problem, both based on the partial ability of cholesterol to replace ergosterol as growth factor for the yeast auxotroph GL7.

At certain levels of sterol supplement, e.g., 0.5 μg/ml, the mutant grows about half as rapidly on cholesterol as it does on ergosterol (4), the principal sterol that wild type yeast normally produces. These differences in growth rates have enabled us to demonstrate, first of all, synergistic sterol effects on eukaryotic cell growth and also on oleate incorporation into yeast phospholipids (4). Secondly, with the aid of the "shift up" technique, Dahl and Dahl (15) have shown very marked sterol effects on PI phosphorylation and stimulation of a membrane protein kinase activity (7).

The observations that ergosterol accelerates the methylation of PE to PC (6) were made by comparing biosynthetic rates both in vivo and in memebrane preparations derived from cells grown separately on ergosterol and cholesterol, respectively, to the same absorbance. We have used the same methodology, i.e., membrane preparations derived from ergosterol- or cholesterol-supplemented cells to ascertain whether sterol control of phospholipid biosynthesis is a general or a selective phenomenon. We now show that, in addition to PC synthesis by PE methylation, α-glycerol-phosphate acylation is also sterol-stimulated, while four other phospholipid biosynthetic processes are not. Moreover, the polyene antibiotic filipin inhibits only those processes that ergosterol stimulates.

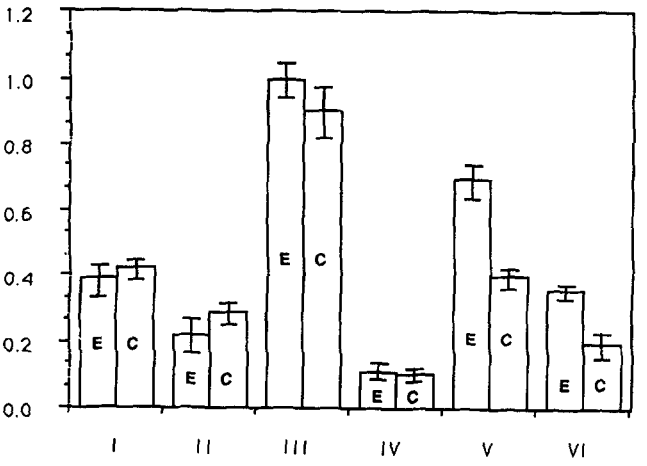

Fig. 1. Specific enzyme activities, nmol of product/min per mg protein, of membrane fractions from yeast mutant strain GL7. I. Long-chain acyl CoA synthetase; II. CTP-phosphatidate cytidyl transferase; III. phosphatidylserine decarboxylase; IV. phosphatidylinositol synthetase; V. acyl-CoA-α-glycerol-3-phosphate transacylase; VI. phosphatidylethanolamine-phosphatidylcholine N-methyl transferase; E, cells grown on 1.0 μg/ml of ergosterol or C, cells grown on 1.0 μg/ml of cholesterol, both to an optical density (A_{540}) of 0.3–0.4. Membranes were prepared as described in Materials and Methods.

Fig. 1 compares the specific activities of various phospholipid biosynthetic reactions tested in membrane protein derived from cells grown on ergosterol or cholesterol, respectively, under as nearly identical conditions as possible. In all cases, the cells that served as enzyme sources were harvested in the mid-log phase (A_{540} about 0.4). This point deserves to be stressed in view of the report (16) that in some instances, e.g., phospholipid

TABLE 1. PE→PC transmethylase activities of intact cells grown either on ergosterol (Erg) or cholesterol (Chol) as a function of cell density at time of harvest

Incubation Time with [Methyl-^{3}H]-Methionine	Absorbance (A_{450}) at Harvest	Experiment 1		Experiment 2		Experiment 3		E/C[a]
		Erg	Chol	Erg	Chol	Erg	Chol	
min		*incorporation of [methyl-^{3}H]methionine into PC nmol/mg protein*						
10	0.25	0.020	0.015	0.014	0.007	0.068	0.020	2.24
10	0.55	0.017	0.010	0.031	0.022	0.080	0.060	1.48
10	1.20			0.60	0.058	0.05	0.060	0.93
20	0.25	0.036	0.025	0.042	0.012			2.47
20	0.55	0.062	0.032	0.062	0.042	0.040	0.020	1.80
20	1.20			0.170	0.160	0.184	0.120	1.30
30	0.25	0.080	0.042		0.018			[1.91]
30	0.55	0.100	0.076	0.090	0.066	0.080	0.050	1.43
30	1.20			0.280	0.260	0.380	0.340	1.1

[a]Ratios of PE →PC transmethylase activities were averages of two or three experiments as indicated, and bracketed when only a single experiment was available.

TABLE 2. Acyl-CoA-glycerol-3-phosphate acyl transferase activities of yeast membrane fractions

Exp.	Phosphatidic Acid		E/C	Lysophosphatidic Acid		E/C
	Erg	Chol		Erg	Chol	
	nmol/min/mg protein			*nmol/min/mg protein*		
I	0.55	0.35	1.58	3.34	2.72	1.23
II	0.87	0.45	1.93	2.44	2.33	1.05
III	1.01	0.7	1.44	2.81	2.40	1.17
Average			1.65			1.15

Oleyl CoA was the substrate in experiments I and II, and palmitoyl CoA was the substrate in experiment III. Cells were grown on 1 μg/ml of ergosterol (Erg) or cholesterol (Chol), respectively, and harvested at an absorbance of 0.3. The numbers given are averages of duplicates. For further details, see Materials and Methods.

N-methyl transferase, enzyme activity declines sharply (two- to fivefold) when yeast (wild type) cells enter the stationary phase, while in others growth phase at time of harvest is without effect on biosynthetic rates. We have checked this particular point only for phospholipid N-methyl transferase from GL7 and find that in this instance the magnitude of the E/C ratio (specific activity ratio for enzymes derived, respectively, from ergosterol- or cholesterol-supplemented cells) is highest by far in the early log phase (absorbance = 0.25), intermediate in mid-log phase (absorbance = 0.55) and close to unity in stationary phase cells (absorbance = 1.20) (**Table 1**). For technical reasons we have not obtained such information for the other enzymes tested here. We believe, however, that determination of all specific enzyme activities from cells harvested at identical or near identical A_{540} values (about 0.4) is the nearest we can come to a valid comparison (Fig. 1). With this proviso we conclude that the superior activity of ergosterol as a growth factor for the yeast mutant GL7 is in part attributable to its regulatory effects on selected, perhaps rate-limiting, steps in phospholipid biosynthesis. PC synthesis by PE methylation may be in this category, especially since the transmethylation pathway predominates over the alternate route to PC by way of phosphocholine in wild type yeast and even more markedly in the yeast strain GL7 (17). It is of interest that according to preliminary results (J. Chin and K. Bloch, unpublished) the reactions of the alternate pathway to PC by way of phosphocholine occur at the same rate in GL7 cells grown either on ergosterol or cholesterol. Whether the one other enyzme we have found to respond to ergosterol, acyl CoA-glycerophosphate transacylase (**Table 2**), is rate-limiting is not known either for wild type yeast or the mutant GL7.

Most likely two separate enzymes catalyze glycerol esterification at C_1 and C_2, respectively. Our results for lysophosphatidic acid formation, obtained with total membrane fractions (Table 2 and **Table 3**) show 3–6 times greater specific activities for lysophosphatidate than for phosphatidate formation, both in "erg" and in "chol" membrane preparations. However, the E/C ratios, while significantly greater than 1.0 for PA formation, are closer to unity for the synthesis of the lyso compound. This suggests that for PA formation the second acylation step may be rate-limiting, possibly rationalizing sterol control. Similar arguments may apply to the PE–PC transmethylation which also involves several consecutive steps, in this instance the transient monomethyl and dimethyl amino precursors of PC. When these intermediates were isolated (6), their E/C ratios were only slightly greater than unity (average of 1.2), compared to 1.5–2.0 for the overall conversion of PE to PC. In this instance also it is not clear whether and how many separate enzymes (transmethylases) are involved.

TABLE 3. Effect of filipin on acyl CoA-glycerol-3-phosphate transacylase activity of yeast membrane fractions

Additions	Phosphatidic Acid Specific Activity		Lysophosphatidic Specific Activity	
	Erg	Chol	Erg	Chol
	nmol formed /min/mg protein			
None	0.85	0.53	2.75	2.27
DMSO	0.87	0.51	2.40	2.50
Filipin, 0.25 mM	0.56 (64)[a]	0.53 (100)	2.45 (100)	2.41 (96)
Filipin, 0.5 mM	0.34 (39)	0.66 (124)	2.15 (89)	2.46 (100)
Filipin, 0.75 mM	0.28 (32)	0.40 (78)	1.56 (64)	1.84 (74)
Filipin, 1.0 mM	0.36 (41)	0.28 (53)	1.39 (58)	1.42 (57)

Cells were grown in the presence of either 1 μg/ml of ergosterol (Erg) or cholesterol (Chol) to an absorbance of 0.3. Enzyme activity was assayed after 10 min incubation. Filipin solutions contained 25 μl/ml of DMSO.
[a]Numbers in parentheses, % of control specific activity.

TABLE 4. Effect of filipin on oleyl CoA synthetase activity of cells grown on either ergosterol or cholesterol

Addition	Ergosterol		Cholesterol	
	Specific Activity	% of Control	Specific Activity	% of Control
	nmol/min/mg protein			
None	0.35		0.32	
Filipin, 0.25 mM	0.41	120	0.46	145
Filipin, 0.50 mM	0.38	110	0.48	150

All samples contained 25 μg/ml of DMSO. This solvent alone inhibits enzyme activity by about 10%. Data are averages for duplicate or triplicate observations. For details, see Materials and Methods.

Filipin inhibition

Effects of the sterol-complexing polyene antibiotics on in vitro systems have been examined only rarely. We have previously found (6) that these agents are reasonably potent inhibitors of the overall PE-PC conversion albeit at fairly high concentrations (about 50% at 0.5–0.75 mM). By contrast, filipin and amphotericin were without effect on the synthesis of the transient intermediates monomethyl- and dimethyl-PE or slightly stimulated these steps (6).

In the synthesis of phosphatidate also, filipin inhibits the overall process significantly (60–70% at and above 50 mM) while the first acylation, to lysophosphatidic acid, tends to be less filipin-sensitive (10% inhibition at 0.5 mM) (Table 3).

The notion of a linkage between E/C ratios significantly greater than unity and sensitivity to filipin receives further support from the uniform resistance to filipin of acyl CoA synthetase (**Table 4**), CTP-phosphatidate cytidyl transferase (**Table 5**), PS-decarboxylase (**Table 6**), and PI synthetase (data not shown). In fact, some of the reactions in this category, all of which show identical activities in "erg" and "chol" cells, are significantly stimulated by filipin, as shown, for example, in Table 4. Collectively, these correlations strengthen the notion that the ergosterol effects are selective and more difficult to explain by a generalized sterol-induced change, e.g., of the physical state of the yeast membrane.

As mentioned earlier, Dahl and Dahl (15) have observed some striking ergosterol effects on poly-phosphoinositide turnover as well as on budding and growth of sterol-starved yeast and secondly, a dramatic stimulation of a yeast kinase by as little as 10^{-8} M ergosterol (7). These are first of all very prompt effects and secondly not dependent on protein synthesis, i.e., unchanged by cycloheximide. For the two sterol-sensitive reactions we describe here the mechanism of response is likely to be different. In some preliminary experiments under "shift up" conditions such as were used for demonstrating the ergosterol effect on poly-phosphoinositide turnover (15), cycloheximide stopped PC synthesis completely whether or not ergosterol was added to cells growing on cholesterol (M. Ramgopal and K. Bloch, unpublished results). Approaches other than those described here will therefore be necessary to clarify the mechanism of sterol control in phospholipid biosynthesis.

The fraction of the total sterol the mutant GL7 needs for the several distinguishable functions can only be roughly estimated. We suggested that these quantities are smallest for the effects on protein kinase activity in sterol-starved cells (7), intermediate for the control of phospholipid biosynthesis described here and for phosphoinositide turnover (15), and most likely largest by far for the so-called bulk function, i.e., the control of the membrane's physical state. These estimates are also in accord with the results of Parks and collaborators (18, 19) describing the "sparking" effects of ergosterol in yeast mutants that fail to grow with large supplements of cholestanol alone.

Finally, we wish to emphasize that, as far as structural specificity is concerned, the requirement of yeast and yeast mutants for ergosterol is not absolute. Cholesterol and 7-dehydrocholesterol (but not cholestanol) also sup-

TABLE 5. Effect of filipin on CTP-phosphatidate-cytidyl transferase activity in membrane fractions of cells grown on either ergosterol or cholesterol

Addition	Ergosterol		Cholesterol	
	Specific Activity	% of Control	Specific Activity	% of Control
	nmol/min/mg protein			
None	0.19		0.24	
Filipin, 0.25 mM	0.20	95	0.26	110
Filipin, 0.50 mM	0.22	105	0.31	130

All samples contained 25 μg/ml of DMSO. This solvent alone inhibits enzyme activity by about 10%. Data given are averages for duplicate or triplicate observations. For details, see Materials and Methods.

TABLE 6. Effect of filipin on phosphatidylserine decarboxylase activity of membrane fractions from cells grown on either ergosterol or cholesterol

Addition	Ergosterol		Cholesterol	
	Specific Activity	% of Control	Specific Activity	% of Control
	nmol/min/mg protein			
None	0.98		0.90	
Filipin, 0.25 mM	0.96	100	0.92	105
Filipin, 0.50 mM	0.94	96	1.03	120

All samples contained 25 μg/ml of DMSO. For details, see Materials and Methods.

port the growth of strain GL7 albeit less effectivley. The differences are quantitative, not qualitative. This means that any sterol that supports growth, to whatever extent, can satisfy every one of the individual physiological demands of the yeast cell but obviously to varying degrees. Most likely for the "signal" function, the structural requirements are the strictest and the quantities (as fraction of total sterol) the smallest. On the other hand, for the bulk or nonmetabolic function, structural specificity is very much broader and may even be met in some microbes by nonsteroidal squalene cyclization products such as the pentacyclic triterpene tetrahymanol (20, 21).

A summary account of the results reported here has recently appeared (22).

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