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Phenethyl Alcohol Inhibition of *sn*-Glycerol 3-Phosphate Acylation in *Escherichia coli*

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In vivo and in vitro experiments were performed to determine how phenethyl alcohol (PEA) inhibits phospholipid synthesis in *Escherichia coli*. This drug drastically reduced the rate of incorporation of *sn*-glycerol 3-phosphate into the phospholipids of an *sn*-glycerol 3-phosphate auxotroph. PEA also reduced the rate of fatty acid incorporation into the phospholipids of a fatty acid auxotroph. The kinetics of PEA inhibition of the rate of incorporation of *sn*-glycerol 3-phosphate were almost identical to those of PEA inhibition of the rate of fatty acid incorporation into phospholipids. The in vivo experiments suggested that the rate-limiting step(s) in phospholipid biosynthesis inhibited by PEA is at the level of the acylation of *sn*-glycerol 3-phosphate or beyond this step. PEA inhibited the *sn*-glycerol 3-phosphate acyltransferase with either palmitoyl coenzyme A or palmitoyl-acyl carrier protein as the acyl donor. This drug, however, had no effect on the cytidine 5'-diphosphate-diglyceride:glycerol 3-phosphate phosphatidyl transferase, cytidine 5'-diphosphate-diglyceride:L-serine phosphatidyl transferase, and acyl coenzyme A:lysophatidic acid acyltransferase. The in vitro findings suggested that PEA inhibits phospholipid synthesis primarily at the level of *sn*-glycerol 3-phosphate acyltransferase.

The incorporation of labeled precursors into membrane phospholipids of *Escherichia coli* is drastically inhibited by phenethyl alcohol (PEA) (1, 22, 25, 33). Studies in our laboratory have shown that phospholipid synthesis is more sensitive to inhibition by low concentrations of PEA than are deoxyribonucleic acid, ribonucleic acid, and protein syntheses (25). We have also shown that PEA inhibition of phospholipid synthesis is not a secondary effect resulting from the inhibition of macromolecular synthesis (25).

Treatment of *E. coli* with PEA drastically alters the phospholipid composition of the cell membrane (1, 22, 25, 33). The changes in phospholipid composition are due to the inhibitory effect of PEA on the de novo rates of synthesis of phosphatidylethanolamine and phosphatidylglycerol (22). PEA also alters the fatty acid composition of membrane phospholipids by differentially inhibiting the de novo rates of synthesis of saturated and unsaturated fatty acids (22).

Recently, evidence was presented that PEA inhibits total fatty acid synthesis as a secondary consequence of its inhibition of phospholipid synthesis (W. D. Nunn, Biochemistry, in press). We have shown that supplementation of PEA-treated cells with fatty acids or *sn*-glycerol 3-phosphate (G3P), or both, does not reverse the inhibitory effect of the drug on phospholipid biosynthesis (22). These experiments indicated that PEA does not inhibit phospholipid synthesis by limiting the supply of fatty acids or G3P, suggesting that the rate-limiting step(s) in phospholipid synthesis controlled by PEA is at the level of the acylation of G3P or beyond this step.

In this paper, we report the results of in vivo and in vitro experiments designed to assess the site of PEA control of phospholipid synthesis. The in vivo studies showed that PEA reduced the rate of incorporation of G3P into phospholipids to the same degree that it reduced the rate of incorporation of fatty acids into phospholipids, suggesting that PEA inhibited the esterification of fatty acids to G3P. The in vitro studies revealed that PEA inhibited the activity of the G3P acyltransferase with either palmitoyl coenzyme A (CoA) or palmitoly-acyl carrier protein (ACP) as the acyl donor. The drug did not appreciably affect the activity of several other phospholipid biosynthetic enzymes. The in vivo and in vitro results suggested that PEA

exerted its inhibitory effect on phospholipid synthesis at the level of the G3P acyltransferase.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin; DL-glycerol 3-phosphate, disodium salt; acetyl CoA; glucose 6-phosphate; nicotinamide adenine dinucleotide phosphate, reduced form; CoA; *cis*-vaccenyl chloride, Triton X-100 (octylphenoxypolyethoxyethanol); malonyl CoA; palmitic acid; cytidine 5'-triphosphate (CTP); tris(hydroxymethyl)aminomethane (Tris); and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from the Sigma Chemical Co., St. Louis, Mo. DL-[3-^{14}C]serine was a product of the ICN Corp., Irvine, Calif. [^{14}C]G3P, [1-^{14}C]palmitic acid, [9,10-^{3}H]palmitic acid, [^{32}P]orthophosphoric acid, and [2-^{14}C]malonyl CoA were purchased from New England Nuclear Corp., Boston, Mass. Cytosine 5'-diphosphate (CDP)-diglycerides, dioleolyl phosphatidic acid, and *cis*-vaccenic acid were purchased from Serdary Research Laboratories, Ontario, Canada. [5'-^{3}H]CTP was purchased from Schwarz/Mann, Orangeburg, N.Y. PEA was purchased from Matheson Coleman and Bell Co., Rutherford, N.J.

Palmitoyl CoA was synthesized by the method of Seubert (29). *cis*-Vaccenyl CoA was synthesized by the same method but as modified by Pieringer et al. (26). Palmitoyl ACP was prepared by the procedure of Ray and Cronan (27).

Bacterial strains. Bacterial strains used in this study are derivatives of *E. coli* K-12. Their derivation and complete genotypes were described previously (2, 16, 28). Strain 8 (*glpD$^-$ glpR^c phoA$^-$*) was obtained from E.C.C. Lin (16). Strain BB20-14 (kindly provided by R. Bell) is a G3P auxotroph derived from strain 8 and is defective in the anabolic G3P dehydrogenase (2). Strain LA-289 is a temperature-sensitive fatty acid auxotroph that requires both saturated and unsaturated fatty acids for growth at 37°C (28). Its phenotype is due to the thermolability of malonyl CoA-ACP transacylase (28).

Bacteria were routinely incubated in a New Brunswick gyratory water bath-shaker at 37°C. The bacteria were usually cultured in standard medium consisting of medium E (35) supplemented with 0.4% sodium succinate and 0.05% casein hydrolysate (vitamin free). In some experiments, the bacteria were cultured in the synthetic medium of Davis and Mingioli (13). When appropriate, G3P was added to standard medium to a final concentration of 0.01%. Cell growth was monitored at 540 nm in a Klett-Summerson colorimeter (1 Klett unit is equivalent to 5×10^6 cells per ml). All experiments were initiated when the density of an exponentially growing culture reached 2.5×10^8 to 3.0×10^8 cells per ml.

Assay of phospholipid synthesis in vivo. The rate of phospholipid synthesis in strain BB20-14 was determined with [^{14}C]G3P as a precursor. A culture of BB20-14 was grown at 37°C in standard medium (35) supplemented with 0.01% G3P. When a density of 2.5×10^8 cells per ml was reached, the culture was divided into three equal portions. PEA at final concentrations of 4 and 8 mM was added to two portions, and the last portion was left untreated. The cultures were incubated at 37°C, and at the indicated times 1-ml samples were removed and incubated at 37°C for 5 min with 1 μCi of [U-^{14}C]G3P (1.72 μCi/μmol). After the 5 min of incubation, 6 ml of chloroform-methanol (1:2, vol/vol) was added to the tube, and the lipids were treated as described by Nunn and Cronan (23, 24). Radioactivity in the total lipids was quantitated as described previously (23).

Assay of fatty acid incorporation into phospholipids. Cultures of strain LA-289 were grown at 37°C in standard medium supplemented with G3P (4 mg/ml), potassium *cis*-vaccenate (50 μg/ml), potassium palmitate (50 μg/ml), potassium glutamate (1 mg/ml), yeast extract (1 μg/ml), thiamine (0.05 μg/ml), and Brij 58 (4 mg/ml). When a density of 2.5×10^8 cells per ml was reached, the cultures were divided into two equal portions. PEA at a final concentration of 4 or 8 mM was added to one portion. The other portion was left untreated. At various time intervals after the addition of PEA, a 1-ml portion was removed from each culture and placed in a tube containing [9,10-^{3}H]palmitic acid (5×10^6 dpm/ml) and *cis*-[^{14}C]vaccenate (5×10^4 dpm/ml, labeled in the odd-numbered carbons). After 5 min of incubation at 37°C, 6 ml of chloroform-methanol (1:2, vol/vol) was added to each tube, and the lipids were extracted as described by Nunn and Cronan (23). The extracted lipids were then resolved into phospholipid and fatty acid fractions by thin-layer chromatography on silica gel plates developed in diethyl ether-acetic acid (100:1, vol/vol). The phospholipid fraction was counted in a scintillation counter adjusted for dual-label counting. The reciprocal experiment with [^{14}C]palmitate and *cis*-[^{3}H]vaccenic acid gave similar results. The labeled *cis*-vaccenate samples were made by biosynthetic incorporation of labeled acetate as described by Cronan and Batchelor (9).

Assay of $^{32}P_i$ incorporation into nucleotides. The intracellular nucleotide concentrations were determined by the procedure of Cashel et al. (5). Cultures of strain BB20-14 were grown in low-phosphate medium (2) supplemented with 0.4% sodium succinate and 0.01% G3P. When a cell density of 2.5×10^8 cells per ml was reached, the cultures were divided into three equal portions. PEA at final concentrations of 4 and 8 mM was added to two portions, and the last portion was left untreated. Each culture was then labeled with 200 μCi of ^{32}P-labeled inorganic phosphate ($^{32}P_i$; 200 μCi/μmol) per ml. The cultures were incubated at 37°C, and at the indicated times samples (2 ml) were removed and filtered on a membrane filter (Millipore Corp., type HA). The filter and cells were then suspended in an equal volume of ice-cold 2 N formic acid. A portion of the filtrate was also mixed with an equal volume of 2 N formic acid. The nucleotide levels were determined by chromatographing 10-μl volumes of these extracts on polyethyleneimine plates (5). The plates were developed with 0.85 M potassium phosphate buffer (pH 3.4). ^{32}P-labeled nucleotides were located by their cochromatography with unlabeled nucleotide standards, which were located with ultraviolet light.

The extracellular levels of nucleotides were obtained from the assay on the filtrate.

Assay of [^{14}C]G3P uptake. Rates of uptake were measured by the addition of 2.0 ml of a culture of strain BB20-14 to tubes containing 2 μCi of [^{14}C]G3P (1.72 μCi/μmol). The tubes were incubated at 30°C, and at various time intervals 0.2-ml portions were removed, and the cells were collected on Millipore membrane filters (0.45-μm pore size, 25-mm diameter). The cells on the filter were then washed with 10 ml of low-phosphate medium (2). The filtration and washing were completed within 10 s. The filter disks were dried and counted. To show that G3P transport is energy dependent, we treated a portion of the culture with 20 mM sodium azide.

To study the effect of PEA on the leakage of [^{14}C]G3P from the cell, we labeled strain BB20-14 with 1 μCi of [^{14}C]G3P (1.72 μCi/μmol) per ml for 5 min. The cells were then filtered and suspended in the same medium minus labeled [^{14}C]G3P. The cells were next incubated at 37°C in the presence of PEA. At various time intervals, samples were removed and filtered on Millipore membrane filters. The radioactivity in a portion of the filtrate and on the filter disk was determined by scintillation counting.

Assay of beta-oxidation. The beta-oxidation of exogenous fatty acids by intact cells of *E. coli* provides a sensitive indication of the rate of fatty acid transport (and/or activation). The rate of beta-oxidation is more sensitive to such a decrease than is the rate of incorporation of fatty acids into phospholipids (8, 18). Cultures of strain LA2-89 were grown for several generations in medium E (35) supplemented with 0.4% sodium succinate, potassium oleate (0.2 mM), potassium palmitate (0.2 mM), potassium glutamate (1 mg/ml), yeast extract (1 μg/ml), thiamine (0.05 μg/ml), and Brij 58 (4 mg/ml). When a density of 2×10^8 cells per ml was reached, the culture was centrifuged, suspended in minimal medium containing 1% Brij 58, and incubated at 37°C for 120 min. The culture was divided into three equal portions. PEA at final concentrations of 4 and 8 mM was added to two portions, and the last portion was left untreated. All three cultures were supplemented with 0.2 mM [1-^{14}C]oleate (3 nCi/μmol) and 0.2 mM palmitate. The cultures were incubated in a sealed flask (18), and then the reaction was halted by the addition of acid. The CO_2 produced by each culture was collected into Hyamine as described by Klein et al. (18). Comparable results were obtained when [^{14}C]palmitate was used. Under these experimental conditions, PEA inhibited the incorporation of fatty acids into phospholipids to the same extent as observed under the experimental conditions described in Table 4.

Enzyme preparations and assays. Cell extracts from strain 8 were used for assaying fatty acid synthetase and CDP-diglyceride:L-serine phosphatidyltransferase activity. These extracts were prepared as described by Gelmann and Cronan (14). The particulate fractions from strain 8 were prepared as described previously (6, 7, 27). Protein concentrations were determined by a microbiuret procedure (21) or by the procedure of Lowry et al. (19).

The assay for the fatty acid synthetase was a modification of that of Silbert and Vagelos (31). The reaction mixture consisted of (per 0.50 ml) 15 nmol of acetyl CoA, 0.1 μmol of nicotinamide adenine dinucleotide phosphate, 1.0 μmol of glucose 6-phosphate, 0.65 U of glucose 6-phosphate dehydrogenase (130 U/mg), 24 μg of freshly reduced ACP, 30 nmol of [2-^{14}C]malonyl CoA (13.4 μCi/μmol), 1.0 μmol of β-mercaptoethanol, and 50 μmol of potassium phosphate buffer (pH 7.0). Reactions were carried out at 37°C in screw-capped tubes sealed with Teflon-lined caps. The reaction was started by the addition of 200 μg of protein from the cell extract and quenched by the addition of 1 ml of 15% KOH in methanol. The samples were acidified to pH 1 with 6 N HCl and extracted twice with petroleum ether. The amount of radioactivity in fatty acids was then quantitated as described previously (14).

The CDP-diglyceride:G3P phosphatidyltransferase activity was assayed by monitoring the conversion of [^{14}C]G3P into chloroform-extractable material as described previously (6, 7). The reaction mixture (final volume, 0.5 ml) for the assay contained 0.25 M Tris-hydrochloride (pH 8.0), 0.08 mM CDP-dipalmitin, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mg of Triton X-100, 0.4 mM [^{14}C]G3P (0.06 μCi/μmol), and 100 to 200 μg of particulate enzyme. The assay was linear with time for at least 30 min over a range of protein concentrations and was fully dependent upon the presence of CDP-diglyceride.

The procedure of Kanfer and Kennedy (17) was used for assaying the CDP-diglyceride:L-serine phosphatidyltransferase. The reaction mixture (final volume, 0.3 ml) contained 0.04 M Tris-hydrochloride (pH 8.0), 0.1 M Na_2SO_4, 10 mM β-mercaptoethanol, 2 mM ethylenediaminetetraacetate, 0.08 mM CDP-dipalmitin, 2 mg of Triton X-100 per ml, 2 mM DL-[3-^{14}C]serine (0.2 μCi/μmol), and 200 to 300 μg of cell extract. The assay was linear for at least 30 min and was fully dependent upon CDP-diglyceride.

The procedure of Carter (4) was used for assaying CTP:phosphatidic acid cytidyltransferase activity. The reaction mixture consisted of 0.16 M potassium phosphate buffer (pH 6.5), 2.0 mM dioleolyl phosphatidic acid, 4.0 mM $MgCl_2$, 1 mM [5'-^{3}H]CTP (8.8 μCi/μmol), and 80 to 250 μg of particulate enzyme per ml. The assay was linear for at least 30 min and was fully dependent upon phosphatidic acid.

The activity of the G3P acyltransferase was measured by the incorporation of [U-^{14}C]G3P into phospholipid. The standard reaction mixture contained 0.1 M Tris-hydrochloride (pH 8.5), 6.25 mM $MgCl_2$, 75 μg of bovine serum albumin (fatty acid free), 97 μM [^{14}C]G3P (25.8 μCi/μmol), 125 μM palmitoyl CoA (or *cis*-vaccenyl CoA) or 70 μM palmitoyl ACP, and 700 μg of particulate enzyme in a final volume of 0.4 ml. The assays were performed at 30°C and were initiated by adding the acyl donor. The reaction was monitored by placing 50 μl of the assay mixture on a Whatman no. 3 MM filter paper disk. The disks were treated as described previously (7). Alternatively, total lipids were extracted from the reaction mixture with chloroform-methanol by the

method of Bligh and Dyer (3). The assay was linear for at least 30 min and was fully dependent upon fatty acyl CoA or fatty acyl ACP.

The activity of the acyl CoA:lysophosphatidic acid acyltransferase was assayed by monitoring the release of CoA resulting from the acyl transfer of oleolyl CoA to lysophosphatidic acid. The appearance of the thiol group of CoA was measured by its reduction of DTNB. The reaction mixture contained (in a final volume of 1 ml) 100 μmol of Tris-hydrochloride (pH 8.5), 0.5 μmol of $MgCl_2$, 0.15 μmol of lysophosphatidic acid, 1.0 μmol of DTNB, 1 mg of bovine serum albumin, 60 nmol of oleolyl CoA, and 60 μg of enzyme preparation. Assays were performed at 25°C and initiated by the addition of lysophosphatidic acid. The assay was linear for at least 30 min and fully dependent upon lysophosphatidic acid.

RESULTS

Effect of PEA on the intracellular levels of nucleotide triphosphates. The experiments reported in this paper were performed with non-bacteriostatic concentrations of PEA (22). It was shown previously that these concentrations, 4 and 8 mM, profoundly inhibit phospholipid synthesis while exerting only a mild effect on macromolecular synthesis (22). The results in Table 1 show that these concentrations of PEA did not significantly affect the intracellular concentration of nucleotide triphosphates. In addition, we found that the extracellular levels of nucleotide triphosphates from untreated and 8 mM PEA-treated cultures are comparable (data not shown). These results suggest that the non-bacteriostatic concentrations of 4 and 8 mM PEA do not cause the cells to become leaky or lose their energy supply.

Effect of PEA on the incorporation of G3P into phospholipids. In our next experiment, we examined the effect of PEA on the rate of [^{14}C]G3P incorporation into the phospholipids of a G3P auxotroph, strain BB20-14. This strain has a defect in the *gpsA* gene, which codes for the biosynthetic G3P dehydrogenase (2), and is therefore deficient in G3P synthesis. Thus, this strain requires exogenously supplied G3P for phospholipid synthesis (and growth).

The results (Table 2) show that 8 mM PEA inhibited the rate of G3P incorporated into phospholipids by approximately 60% and that 8 mM PEA inhibited the uptake of G3P by less than 9%. Cells treated with this concentration of PEA did not appreciably leak G3P into the medium (data not shown). These results suggest that the reduction in the incorporation of G3P into phospholipids caused by PEA (Table 2) cannot be attributed to the inability of the cells to transport G3P. The kinetics of PEA inhibition of the rate of phospholipid synthesis as determined by [^{14}C]G3P incorporation (Table 2) are almost identical to the kinetics of PEA inhibition of phospholipid synthesis as measured by the incorporation of two other radioactive precursors, $^{32}P_i$ and [^{14}C]acetate (22). These results (Table 2) indicate that PEA controls phospholipid synthesis beyond the level of G3P synthesis.

The reduction in the rate of G3P incorporation into phospholipids caused by PEA may be due to one or more of the following reasons: (i) PEA inhibits fatty acid synthesis and, thereby, reduces the amount of fatty acids available for esterification to G3P; (ii) PEA inhibits the acylation of G3P by fatty acids; or (iii) PEA inhibits the rate of G3P incorporation into the distal portion of phosphatidylglycerol. The third explanation is unlikely because 8 mM PEA in-

TABLE 1. *Effect of PEA on the intracellular levels of nucleotide triphosphates in strain BB20-14*[a]

PEA concn (mM)	Time (min)	^{32}P incorporated into nucleotide triphosphates (cpm/10-μl culture)			
		GTP	ATP	CTP	UTP
0	15	1,274	2,694	775	1,239
	30	3,733	7,052	2,036	3,232
4	15	1,289	2,647	760	1,240
	30	3,645	6,988	2,011	3,177
8	15	1,221	2,370	740	1,218
	30	3,471	6,603	1,987	2,973

[a] See Materials and Methods for details. GTP, ATP, CTP, UTP, Guanosine, adenosine, cytosine, and uridine 5'-triphosphates, respectively.

TABLE 2. *Effect of PEA on the rate of G3P incorporation into the phospholipids of strain BB20-14*

Conditions	Uptake of G3P in 0.5 min[a] (pmol/10^7 cells)	Rate of G3P incorporation into phospholipids[b] (pmol/min per 10^7 cells)
Untreated	690	28
4 mM PEA	660	16
8 mM PEA	630	11
20 mM sodium azide	30	

[a] Transport experiments were performed at 30°C as described in the text. Data are given for the 30-min time points.

[b] The rate of incorporation of G3P into phospholipids was determined at 37°C as described in the text. Data are given for the 30-min time points.

hibits the rate of phosphatidylglycerol synthesis by about 30% (22), and it inhibits the rate of G3P incorporation into the distal portion of phosphatidyl glycerol by only 15% (W. D. Nunn, unpublished data).

Evidence indicating that PEA does not affect fatty acid synthesis is as follows: (i) PEA does not inhibit the accumulation of free fatty acids in beta-oxidation-less G3P auxotrophs starved of G3P (22); and (ii) PEA at a final concentration of 8 mM inhibits the in vitro activity of the fatty acid synthetase by less than 5% (data not shown). These results suggest that it is unlikely that the reduction of G3P incorporation into phospholipids caused by PEA (Table 2) is due to limiting levels of fatty acids. To further substantiate the evidence that PEA does not inhibit phospholipid synthesis by limiting the supply of fatty acids available for esterification to G3P, we determined the effect of this drug on phospholipid synthesis in bacteria supplemented with both fatty acids and G3P.

To ensure that the bacteria utilized the fatty acids, we performed our experiment with a strain deficient in the synthesis of both saturated and unsaturated fatty acids. The strain, LA2-89, synthesizes fatty acids at about one-fourth the normal rate (at 37°C) due to a defect in the malonyl CoA-ACP transacylase (28). Hence, this strain requires supplementation with both an unsaturated and a saturated fatty acid to allow phospholipid synthesis (and growth) to proceed at 37°C. To ensure that the bacteria utilized G3P, we grew strain LA-289 on G3P as the sole carbon and energy source.

As shown in Table 3, 8 mM PEA reduced the rate of incorporation of total fatty acids (both saturated and unsaturated) into the phospholipids of strain LA2-89 by 60%. The incorporation of palmitic acid and *cis*-vaccenic acid into phospholipids was inhibited by this drug to the same extent (Table 3). Since these results could be due to the inhibition of fatty acid transport and/or activation, we determined the effect of PEA on the rate of beta-oxidation in these cells. The rationale for this experiment is that beta-oxidation of exogenous fatty acids by intact cells is more sensitive to changes in fatty acid transport and/or activation than is the incorporation of fatty acids into phospholipids (8, 18). The results (Table 4) show that 8 mM PEA did not appreciably affect the rate of beta-oxidation. The latter results suggest that PEA does not inhibit the incorporation of fatty acids into phospholipids by inhibiting fatty acid transport and/or activation. Since the experiments described in Table 3 were performed with G3P as the sole carbon source, these experiments indicate that PEA controls phospholipid synthesis beyond both fatty acid and G3P synthesis.

TABLE 3. *Effect of PEA on the incorporation of fatty acids into phospholipids in E. coli*[a]

PEA concn (mM)	Time (min)	Rate of fatty acid incorporation into phospholipids (pmol of fatty acid/min per 10^7 cells)		
		Total	Saturated	Unsaturated
0	15	55	28	27
	30	54	27	27
4	15	34	18	16
	30	32	18	14
8	15	25	12	13
	30	21	11	10

[a] See Materials and Methods for details.

TABLE 4. *Effect of PEA on the oxidation of exogenous fatty acids in strain LA2-89*[a]

PEA concn (mM)	Rate of CO_2 production (nmol/min per mg of protein)
0	55.2
4	52.3
8	49.2

[a] See Materials and Methods for details.

The results in Table 2 and 3 also reveal that PEA inhibited the incorporation of approximately 2.0 mol of fatty acid per mol of G3P into phospholipids. Since the molar ratio of fatty acids to G3P in the phospholipids of PEA-treated cells is also approximately 2.0 (Table 2 and 3), the latter finding suggests that PEA might be controlling phospholipid synthesis by inhibiting the esterification of fatty acids to G3P.

Effect of PEA on the G3P acyltransferase. To test if PEA inhibits phospholipid synthesis at the level of the acylation of G3P, we determined the effect of PEA on the in vitro activity of the G3P acyltransferase. The results (Table 5) show that 8 mM PEA inhibited the activity of the G3P acyltransferase by approximately 50% when palmitoyl CoA was used as acyl donor and by about 60% when *cis*-vaccenyl CoA was used as acyl donor.

Since fatty acyl ACP is considered to be the in vivo acyl donor utilized for de novo phospholipid synthesis (20), we compared the effects of PEA on the G3P acyltransferase with either native palmitoyl ACP or palmitoyl CoA as the acyl donor. The results (Table 6) show that 8 mM PEA reduced the activity of the G3P acyltransferase by about 50% when palmitoyl CoA

TABLE 5. *Effect of PEA on the acylation of G3P*[a]

Acyl donor	PEA concn (mM)	G3P incorporated (nmol/min per mg of protein)	Activity (%)
Palmitoyl CoA	0	1.13	100
	4	0.77	69
	8	0.58	51
cis-Vaccenyl CoA	0	0.53	100
	4	0.31	59
	8	0.22	42

[a] The assay conditions for the G3P acyltransferase were the same as described in Materials and Methods.

TABLE 6. *Effect of PEA on the G3P acyltransferase with acyl ACP and acyl CoA as acyl donors*[a]

Acyl donor	PEA concn (mM)	G3P incorporated (nmol/min per mg of protein)	Activity (%)
Palmitoyl CoA	0	4.2	100
	8	2.1	51
Palmitoyl ACP	0	3.9	100
	8	1.7	43

[a] The assay conditions were the same as that described in the text, except that 200 μM G3P and 50 μM palmitoyl CoA or 70 μM palmitoyl ACP were used in the reaction mixture. The enzyme preparations used in the experiments described in this table and in Table 7 were somewhat more active than that used for the experiments presented in Table 5.

was the acyl donor and by 57% when palmitoyl ACP was the acyl donor.

The results in Table 7 indicate that the inhibitory effect of PEA on the acyltransferase was significant over the concentration ranges of either palmitoyl CoA or G3P used. The apparent K_m for G3P obtained from these studies was 1.5×10^{-4} M, and the V_{max} was 6.3 nmol/min per mg of protein. PEA did not alter the K_m for G3P, but it did decrease the V_{max} to 3.3 nmol/min per mg of protein. These data suggest that PEA acts as a noncompetitive inhibitor with respect to G3P. Additional kinetic studies on the effect of PEA on the acyltransferase with respect to palmitoyl CoA show that the apparent K_m (5.0×10^{-5} M) and the V_{max} (7.0 nmol/min per mg of protein) were both decreased by the addition of PEA (data not shown). Although these results suggest uncompetitive or "mixed" inhibition by PEA, more thorough kinetic studies are required to prove this conclusively. One difficulty in performing such experiments is the fact that palmitoyl CoA at concentrations greater than 7.5×10^{-5} M is inhibitory to the acyltransferase. The inhibitory effect of PEA on the acyltransferase is reversible by dilution (data not shown). These in vitro experiments (Tables 5 through 7) suggest that one site of PEA control of phospholipid synthesis is at the level of the acylation of G3P.

Effect of PEA on other phospholipid biosynthetic enzymes. Since our earlier in vivo studies (25) showed that PEA inhibits the synthesis of phosphatidylglycerol and phosphatidylethanolamine, we decided to determine the effect of this drug on the CDP-diglyceride:G3P phosphatidyltransferase (phosphatidylglycerophosphate synthetase) and on the CDP-diglyceride:L-serine phosphatidyltransferase (phosphatidylserine synthetase). The results (Table 8) show that PEA, at concentrations of 8 and 16 mM, had a negligible effect on the phosphatidylglycerophosphate and phosphatidylserine synthetases. PEA also had no effect on the in vitro activity of CTP:phosphatidic acid cytidyltransferase (Table 8) and the acyl CoA:lysophosphatidic acid acyltransferase (Table 8).

DISCUSSION

In our earliest studies, we characterized the effect of PEA on phospholipid metabolism in *E. coli* (22, 25). We showed that PEA inhibits the rate of phospholipid synthesis, and this inhibitory effect is not a secondary consequence of macromolecular synthesis (25). We also showed that the inhibitory effect of this drug on phospholipid synthesis cannot be reversed by sup-

TABLE 7. *Effect of PEA on the G3P acyltransferase at various substrate concentrations*[a]

Variable substrate	Concn of variable substrate (μM)	Untreated: nmol of lipid/min per mg of protein	8 mM PEA: nmol of lipid/min per mg of protein	8 mM PEA: Activity (%)
Palmitoyl CoA[b]	100	4.0	1.9	48
	50	2.5	1.3	52
	25	1.4	0.7	50
G3P[c]	200	3.9	1.8	46
	100	2.5	1.2	48
	50	1.6	0.8	50

[a] The assay conditions were described in Materials and Methods.

[b] A concentration of a 100 μM G3P was used in each reaction mixture.

[c] A concentration of 50 μM palmitoyl CoA was used in each reaction mixture.

TABLE 8. *Effect of PEA on enzymes involved in phospholipid synthesis*[a]

Condition	PEA in assay (mM)	Activity (%)
I. CDP-diglyceride:G3P phosphatidyltransferase	None[b]	100
	8.0	104
II. CDP-diglyceride:L-serine phosphatidyltransferase	None[c]	100
	8.0	97
III. CTP:phosphatidic acid cytidyltransferase	None[d]	100
	16	105
IV. Acyl CoA:lysophosphatidic acid acyltransferase	None[e]	100
	16	100

[a] These assays were performed as described in Materials and Methods.

[b] The CDP-diglyceride:G3P phosphatidyltransferase incorporated 0.70 nmol of G3P per min into chloroform-extractable material.

[c] The CDP-diglyceride:L-serine phosphatidyltransferase incorporated 0.82 nmol of G3P per min into chloroform-extractable material.

[d] The CTP:phosphatidic acid cytidyltransferase incorporated 4.0 pmol of CTP per min into chloroform-extractable material.

[e] The acyl CoA:lysophosphatidic acid acyltransferase reduced 1.5 nmol of DTNB per min.

plementing the PEA-treated cells with fatty acids or G3P, or both (22). The latter results indicate that the site of PEA control on phospholipid synthesis must be beyond the level of fatty acid and G3P synthesis. In our present work, we determined the kinetics of G3P and fatty acid incorporation into the phospholipids of PEA-treated cells. These studies showed that the rate of phospholipid synthesis was inhibited by PEA to the same extent with either of these lipid precursors (Tables 2 and 3). An inspection of the data in Tables 2 and 3 also revealed that PEA reduced the rate of phospholipid synthesis by inhibiting the incorporation of approximately 2 mol of fatty acid per mol of G3P into phospholipids. Our interpretation of these data was that PEA inhibits the esterification of one fatty acid to G3P and, as a consequence, limits the amount of monoacyl G3P (lysophosphatidic acid) available for esterification by a second fatty acid.

Our interpretation is supported by the following results. (i) In vitro studies showed that PEA inhibited the G3P acyltransferase, the enzyme that catalyzes the acylation of fatty acids to G3P (Table 5), indicating that PEA inhibited the activity of this enzyme to almost the same extent that it inhibited the rate of phospholipid synthesis in vivo (Table 2). In addition, the in vitro results showing that PEA inhibited the G3P acyltransferase with unmodified palmitoyl ACP (Table 6) strongly suggest that PEA interferes with the in vivo activity of this enzyme. (ii) In vitro studies showed that PEA did not inhibit the second enzyme in the phospholipid biosynthetic pathway, the acyl CoA:lysophosphatidic acid acyltransferase (Table 8). The lack of accumulation of lysophosphatidic acid in PEA-treated cells (W. D. Nunn and B. E. Tropp, unpublished data) suggests that PEA does not inhibit this enzyme in vivo.

PEA also did not inhibit the in vitro activity of the third enzyme in the phospholipid biosynthetic pathway, the CDP-diglyceride synthetase (Table 8). Since no phosphatidic acid accumulates in PEA-treated cells (Nunn and Tropp, unpublished data), it's unlikely that PEA inhibits the CDP-diglyceride synthetase in vivo.

In earlier studies (22, 25), we reported that PEA inhibits the in vivo synthesis of phosphatidylglycerol and phosphatidylethanolamine. Our in vitro studies on the effect of this drug on the first enzymes in the biosynthetic pathways of phosphatidylglycerol and phosphatidylethanolamine indicate that PEA does not control the synthesis of these phospholipid species at the level of phosphatidylglycerophosphate synthetase and phosphatidylserine synthetase (Table 8). The lack of accumulation of phosphatidylglycerophosphate and phosphatidylserine in PEA-treated cells (Nunn and Tropp, unpublished data) suggests that phosphatidylglycerophosphate phosphatase and phosphatidylserine decarboxylase are not inhibited by this drug in vivo.

We reported earlier (22, 25) that PEA does not inhibit the net synthesis of cardiolipin, the third major phospholipid species in *E. coli*. Evidence indicating that PEA does not control phospholipid synthesis at the level of cardiolipin synthesis was presented in the studies of Tunaitis and Cronan (33), which showed that PEA does not inhibit the in vitro activity of the cardiolipin synthetase. Furthermore, recent studies from this laboratory indicate that cardiolipin continues to be synthesized by *E. coli* even when phosphatidylglycerol formation is inhibited (7, 30, 34).

To date, mutations in two different genes have been shown to affect the activity of the G3P acyltransferase (10, 12). One class of mutations that affect the acyltransferase activity maps at a locus called *plsB* (10). $plsB^-$ strains are characterized by a K_m defect in the G3P acyltransferase (10). The apparent K_m for G3P of the G3P acyltransferase from these strains is 10-fold higher than the K_m of the enzyme from the normal strain (2, 10). A second class of mutations that affect the acyltransferase maps in another locus called *plsA* (12). $plsA^-$ strains have thermolabile acyltransferase activity and also thermolabile adenylate kinase activity (12, 15). Recently, we performed preliminary experiments with a strain carrying two copies of the wild-type acyltransferase $plsB^+$ gene and found

that it was more resistant to inhibition of phospholipid synthesis (and growth) by PEA than strains carrying one copy of the *plsB*$^+$ gene (Nunn, unpublished data). No studies were performed with *plsA*$^+$ strains due to the pleiotropic effects that occur in strains that carry a mutation in this gene (15). However, the preliminary studies with diploid *plsB*$^+$ strains further suggest that PEA affects the activity of the G3P acyltransferase.

PEA is the third drug demonstrated to act at the G3P acyltransferase site. We recently demonstrated that L-glyceraldehyde 3-phosphate and 3-hydroxy-4-oxobutyl-1-phosphonate also inhibit the G3P acyltransferase (32; C. T. Tang, R. Engel, and B. E. Tropp, unpublished data). Unlike PEA, these drugs are competitive inhibitors of G3P in the acyltransferase reaction. L-Glyceraldehyde 3-phosphate is bactericidal, and the phosphonate is bacteriostatic.

In conclusion, these studies suggest that PEA controls phospholipid synthesis by inhibiting the G3P acyltransferase, and the inhibition at this level results in the reduction of the synthesis of phosphatidylglycerol and phosphatidylethanolamine. Since the G3P acyltransferase that is tightly bound to the membrane has, as yet, not been purified, the actual mechanism by which PEA elicits its inhibitory effect on this enzyme remains unknown.

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