Farnesol inhibits phosphatidylcholine biosynthesis in cultured cells by decreasing cholinephosphotransferase activity

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The mechanism of inhibition of phosphatidylcholine (PC) biosynthesis by the isoprenoid farnesol was investigated in the human leukaemic CEM-C1 cell line. Cells were preincubated with 20 μM farnesol for up to 2 h and pulsed with [^{3}H]choline. PC biosynthesis was inhibited to one-quarter at the step catalysed by cholinephosphotransferase (CPT). CPT activity in cellular homogenates from farnesol-treated cells was significantly decreased, but no changes in cytidylyltransferase activity or diacylglycerol concentration were observed. Measurements of CPT activity in the experiments in which farnesol was added directly to the homogenates or microsomal fractions demonstrated that farnesol did not affect CPT activity. However, cytosol from farnesol-treated samples decreased microsomal CPT activity almost twice as much as did cytosol from controls. This effect was found to be heat-stable, and disappeared after dialysis, but could not be attributed to farnesol present in the cytosol. The effect of farnesol was specific when compared with other structurally similar isoprenoids. We conclude that farnesol brings about changes in cultured cells, leading to decreased CPT activity, and thus to the inhibition of PC biosynthesis.

INTRODUCTION

Isoprenoids and their derivatives are widely known as compounds which occupy a key position in biosynthesis of cholesterol, dolichols and ubiquinones. The very important physiologically significant isoprenoid, farnesyl pyrophosphate, is involved in the regulation of the main rate-controlling enzyme of the cholesterogenic pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (for review see [1]). Farnesyl pyrophosphate may be metabolized in the animal liver to farnesyl phosphate, farnesol and farnesoic acid [2]. Both farnesol pyrophosphate and farnesol phosphate have been shown to be involved in protein isoprenylation and in the regulation of cell cycling of different cell lines [3,4].

In our earlier effort to explore the effects of free farnesol on cell growth, we had observed that farnesol added to the cultures of human acute leukaemia CEM-C1 inhibited growth of these cells at concentrations of farnesol in the range 9–30 μM [5].

In the present study we have identified an early biochemical lesion that leads to this growth inhibition. We have shown that farnesol added to the cultures of CEM-C1 cells markedly decreased the activity of CDP-choline: 1,2-diacylglycerol cholinephosphotransferase (PT; EC 2.7.8.2), the last step of phosphatidylcholine (PC) biosynthesis through the Kennedy pathway [6]. This effect was unusual, since most of the agents regulating PC biosynthesis (e.g. phorbol esters, phospholipase C, unsaturated fatty acids, alkylphospholipids and okadaic acid) affect the recognized rate-limiting enzyme CTP: choline-phosphate cytidylyltransferase (CT; EC 2.7.7.15) [7–11]. Experiments carried out as part of this study also revealed that either a metabolic product of farnesol or a heat-stable low-molecular-mass agent induced in response to farnesol treatment was responsible for the inhibitory effect on CPT. The effect of farnesol on PC biosynthesis had certain structural specificity, and most likely did not involve protein isoprenylation.

EXPERIMENTAL

Chemicals

[*methyl*-^{3}H]choline chloride (85 Ci/mmol), phospho[*methyl*-^{14}C]choline (52 mCi/mmol) and CDP-[*methyl*-^{14}C]*choline* (53 mCi/mmol) were purchased from NEN Research Products, Boston, MA, U.S.A.; [γ-^{32}P]ATP was from Amersham Corp., Arlington Heights, IL, U.S.A.

trans-trans-Farnesol, *trans-trans*-farnesyl bromide, *trans-trans*-farnesyl acetate and 3-methyl-but-2-en-1-ol were from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; farnesyl methyl ether was from ICN Biomedicals, Costa Mesa, CA, U.S.A.; and farnesyl pyrophosphate was from American Radiolabeled Chemicals, St. Louis, MO, U.S.A. 1,2-Dioleoyl-*rac*-glycerol and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Cell culture and media

The culture of acute human leukaemia cell line CEM-C1 was a gift from Dr. E. B. Thompson from the University of Texas Medical Branch at Galveston. The cells were propagated routinely in RPMI 1640 medium supplemented with 10% fetal-bovine serum, penicillin (100 units/ml) and streptomycin (100 mg/l). For the experiments, the cells were incubated in serum-free Iscove's medium. Both media were purchased from GIBCO BRL, Gaithersburg, MD, U.S.A. Fetal bovine serum was purchased from Hyclone, Logan, UT, U.S.A.

Treatment with farnesol and other compounds

Cells were preincubated for 24 h in Iscove's medium. After preincubation, cells were counted with a Coulter Model B Counter and adjusted to a density of 2×10^5 cells/ml. The counting procedure developed previously in our laboratory

Abbreviations used: PC, phosphatidylcholine; CT, CTP:choline-phosphate cytidylyltransferase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; DAG, diacylglycerol.

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allowed us to distinguish the number of living cells from the total cell number in suspension [12]. Farnesol or other compounds were added to cell suspensions as ethanol solutions, and the cells were incubated at 37 °C in a 5%-CO_2 atmosphere for various times up to 2 h. Ethanol added to experimental and control samples did not exceed 0.1%. In some experiments involving [^{3}H]choline incorporation into intermediates of PC biosynthesis, Iscove's medium with decreased choline chloride content (0.05 mg/l) was used. This decrease, which allowed us to obtain a higher specific radioactivity of PC precursors, did not influence farnesol effects on PC biosynthesis. In pulse–chase experiments, cells were preincubated in medium containing [^{3}H]choline chloride (2 mCi/l) for 90 min; then medium was replaced with unlabelled medium containing 0.1% ethanol or 20 μM farnesol, and radioactivity was chased with 0.5 mg/l choline for up to 120 min.

In some CPT-activity determinations, farnesol was added directly to the cell homogenate or to microsomal fractions to a final concentration of 20 μM, and the samples were preincubated for up to 1 h at 37 °C before enzyme assay.

Choline incorporation

After treatment, cells were counted and harvested by low-speed centrifugation (500 *g* for 10 min), washed, resuspended in Iscove's medium containing [^{3}H]choline chloride (2 mCi/l) and incubated with gentle agitation at 37 °C for 1 h. Incubation was stopped by centrifugation at 4 °C, and cells were washed three times with ice-cold 1 mM choline chloride in 0.85% NaCl.

Representative samples were transferred to scintillation vials, and their radioactivity was measured to determine [^{3}H]choline incorporation into whole cells. Other samples were subjected to lipid extraction, as described by Bligh and Dyer [13]. Portions of aqueous phase were taken from each sample, freeze-dried and redissolved in 50% methanol, containing unlabelled choline chloride, phosphocholine and CDP-choline. Samples were applied to silica-gel-G-coated chromatographic plates (20 cm × 20 cm; Analtech) and the plates were developed in the solvent 1 M ammonium acetate (pH 5.0)/95% ethanol (3:7, v/v). Spots were revealed by iodine vapour, with choline chloride, phosphocholine and CDP-choline as standards. The spots were scraped and their radioactivity was determined by scintillation counting.

Samples of chloroform phase were dried under nitrogen, redissolved in 30 μl of chloroform containing unlabelled PC and applied to silica-gel-G-coated plates. Plates were developed in the solvent chloroform/methanol/water (27:12:2, by vol.). The spots were revealed by iodine vapour, scraped, and radioactivity was measured by scintillation counting.

Determination of *sn*-1,2-diacylglycerol (DAG) and CDP-choline

After treatment with farnesol for 2 h, cells were harvested by centrifugation, washed three times with ice-cold PBS, and subjected to lipid extraction by the Bligh and Dyer [13] procedure.

Samples of the chloroform fraction were taken from each sample, dried under nitrogen and stored at −20 °C under nitrogen for up to 3 days until assay time. Concentration of *sn*-1,2-DAG in the samples was determined by using the DAG Assay Reagent System (Amersham), in accordance with the original Amersham protocol. Briefly, this assay employs the enzyme DAG kinase, which quantitatively converts *sn*-1,2-DAG into [^{32}P]phosphatidic acid in the presence of [γ-^{32}P]ATP. [^{32}P]Phosphatidic acid was separated by t.l.c., using silica-gel-H-coated glass plates (Analtech). Plates were developed in the solvent chloroform/methanol/acetic acid (13:3:1, by vol.), with unlabelled phosphatidic acid as a standard. The spots were revealed by iodine vapour, scraped and radioactivity was measured by scintillation counting.

After performing lipid extraction by the Bligh and Dyer [13] procedure, samples of aqueous phase were taken, dried and CDP-choline was separated as described above. CDP-choline was determined by estimation of phosphorus content as described by Bartlett [14].

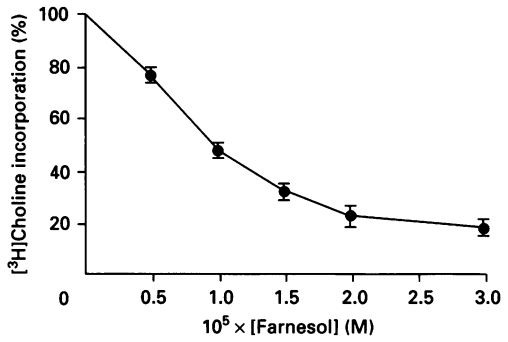


Figure 1 Effect of farnesol concentration on [^{3}H]choline incorporation into lipids of CEM-C1 cells

Cells were preincubated with the indicated concentrations of farnesol for 2 h and then subjected to a 1 h [^{3}H]choline pulse as described in the Experimental section. Cellular lipids were separated by the Bligh and Dyer [13] procedure and the radioactivity was measured by scintillation counting. Data represent percentages of labelled choline incorporation relative to control. Each point represents the mean ± S.D. of three separate incubations. The whole experiment was repeated with essentially the same results.

Cell disruption and fractionation

All procedures were carried out at 4 °C. Pelleted cells were washed three times with Ca^{2+}- and Mg^{2+}-free Earle's salts, resuspended in 1–2 ml of 10 mM Tris/HCl, containing 1 mM EDTA, disrupted with 40 strokes in a Dounce homogenizer and centrifuged at 10000 *g* for 10 min. The supernatant was then centrifuged at 100000 *g* for 60 min (Beckman L5-55 ultracentrifuge, SW56 rotor). The supernatant and pellet obtained refer to the cytosolic and microsomal fractions respectively. The microsomal fraction was resuspended in 10 mM Tris/HCl. Homogenate, microsomal and cytosolic fractions were stored on ice until the time of enzyme assay. Parts of the cytosolic fractions were dialysed against 10 mM Tris/HCl overnight, by using Spectrapor membrane tubing (Spectrum Medical Industries, Los Angeles, CA, U.S.A.).

Enzyme assays

All assays were performed under conditions determined to be linear for time and enzyme concentration.

CT activity was measured as described by Sleight and Kent [8] in the medium containing 20 mM Tris/succinate (pH 7.8), 6 mM $MgCl_2$, 8 mM CTP, 4 mM [^{14}C]phosphocholine (0.4 Ci/mol) and up to 80 μg of protein. Incubation was carried out at 37 °C for 20 min and stopped by immersing samples into boiling water for 2 min. CDP-choline and phosphocholine were separated by t.l.c. as described above. The CDP-choline spots were scraped and their radioactivity was measured by scintillation counting.

CPT was assayed as described by Sleight and Kent [8] in a mixture of 175 mM Tris/HCl (pH 8.5), 8 mM $MgCl_2$, 0.5 mM EGTA, 1 mg/ml BSA and 2 mM DAG in 0.1% taurocholate

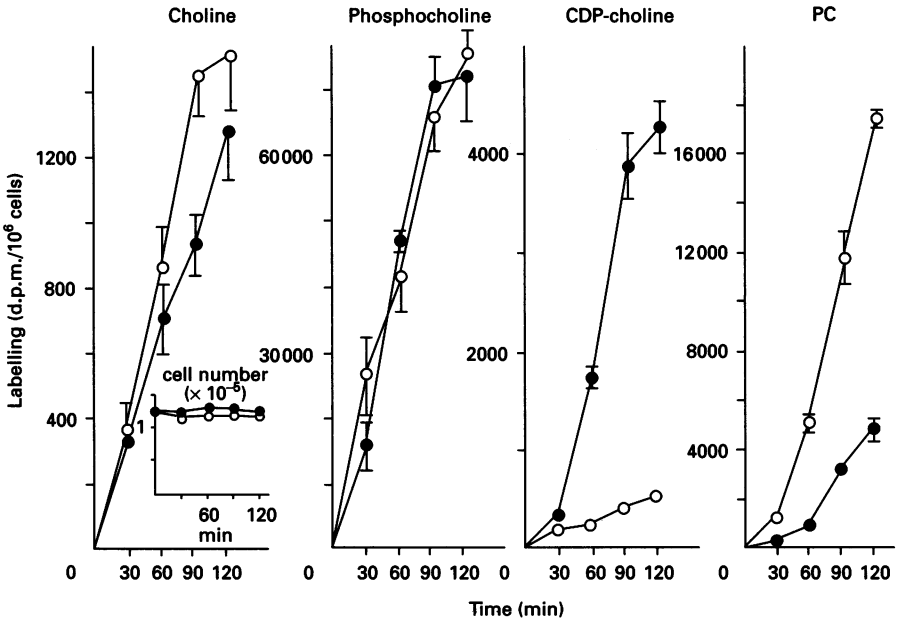


Figure 2 Effect of farnesol on labelling of PC and its precursors

Cells were preincubated with 20 μM farnesol (●) or without farnesol (○) for the indicated times. After the end of incubation, cells were counted for radioactivity, washed with fresh medium and subjected to a 1 h [^{3}H]choline pulse. PC and its precursors were analysed as described in the Experimental section. The values of incorporated radioactivity are given in d.p.m./10^6 cells, as means ± S.D., and represent results of three separate incubations. The whole experiment was repeated with similar results.

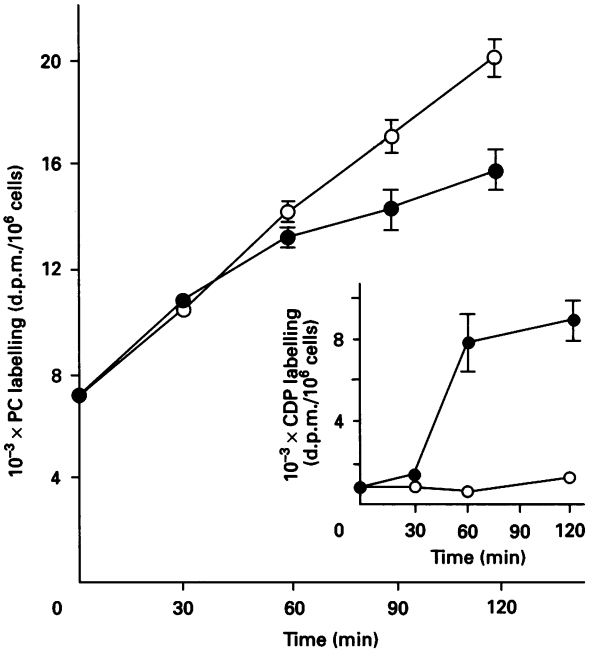


Figure 3 Time-dependent labelling of PC and CDP-choline and farnesol treatment of cells prelabelled with [^{3}H]choline

Cells were preincubated for 90 min with [^{3}H]choline (2 mCi/l) and radioactivity was chased with 0.05 mg/l choline in the absence (○) or presence (●) of 20 μM farnesol. PC and CDP-choline (insert) were separated, and the radioactivity of each was determined as described in the Experimental section. Each point represents the mean ± S.D. of three separate incubations.

(sonicated before addition to assay mixture), 0.1 mM [^{14}C]CDP-choline (2.0 Ci/mol) and up to 25 μg of protein. The reaction was carried out at 37 °C for 8 min and stopped by addition of chloroform/methanol (1:2, v/v). After addition of chloroform and phase separation, the chloroform phase was washed three times with 1% $HClO_4$ and its radioactivity was measured by scintillation counting. Blanks contained all reagents except the enzyme. Radioactivity of blank samples did not exceed background values.

Miscellaneous determinations and measurements

Radioactivity was measured with a Packard model 2200 CA scintillation analyser, by using Packard Poly-Fluor (for aqueous samples) or Opty-Fluor O (for non-aqueous samples) scintillation fluids. Protein was determined by the Lowry method, with BSA as standard.

RESULTS

Effect of farnesol treatment on [^{3}H]choline incorporation into intermediates of PC biosynthesis

As shown in Figure 1, farnesol inhibited [^{3}H]choline incorporation into CEM-C1 cellular lipids in a concentration-dependent manner.

We then determined the amount of [^{3}H]choline incorporated into the different intermediates of the Kennedy pathway. Treatment of cells with 20 μM farnesol decreased radioactive-label incorporation into PC to 25% of the control value (Figure 2). The radiolabel accumulated in the CDP-choline pool, indicated that inhibition of PC biosynthesis had taken place at the level of CPT, the enzyme catalysing the transfer of the choline phosphate moiety from CDP-choline to 1,2-DAG [6]. Incorporation of [^{3}H]choline into other precursors, i.e. choline and phosphocholine, was not affected.

Table 1 Effect of farnesol on CT activity and 1,2-DAG concentration in CEM-C1 cells

Cells were incubated for 2 h in the absence or presence of farnesol (20 μM). Enzyme assay or DAG determination were performed as described in the Experimental section. Data represent means $\pm$ S.D. of three or four (DAG) separate incubations: *differences between control and farnesol-treated samples were not significant in a Student's t test ($P > 0.1$).

	Control	Farnesol
CT activity (nmol/min per mg)		
Homogenate	0.41 $\pm$ 0.07	0.39 $\pm$ 0.05*
Microsomes	0.18 $\pm$ 0.03	0.25 $\pm$ 0.05*
Cytosol	0.17 $\pm$ 0.02	0.14 $\pm$ 0.01*
DAG concn. (pmol/10^7 cells)	336 $\pm$ 36	274 $\pm$ 39*

Table 2 Effect of farnesol on CPT activity in CEM-C1 cells

Farnesol (20 μM) was added to the cell suspension, and cells were incubated for 2 h. The control contained an equivalent amount of ethanol. Homogenate and microsomal fractions were obtained and enzyme assay was performed as described in the Experimental section. In some cases farnesol was added directly to the homogenate or microsomal fraction from control samples before enzyme assay. The results expressed as percentage of control value. Data represent results of six incubations in three experiments; S.D. in these experiments did not exceed 10% of the mean.

	CPT activity (%)	
Sample	Homogenate	Microsomes
Control	100	100
Farnesol added to cell suspension	49	153
Farnesol added to cellular fraction	99	98

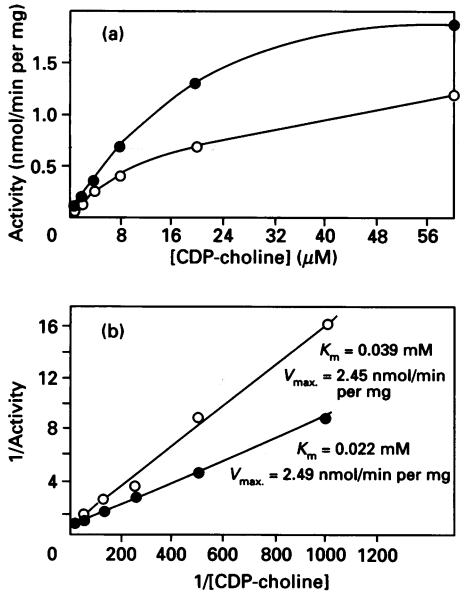


Figure 4 (a) CPT activity in microsomes as a function of CDP-choline concentration; (b) the same data, represented as double-reciprocal plot

Cells were preincubated with 20 μM farnesol (●) or without farnesol (○) for 2 h. The microsomal fraction was prepared and CPT activity was measured as described in the Experimental section. The activity given in nmol/min per mg of protein. Each point represents the mean of two determinations.

To avoid the potential problem of apparent changes in the labelling of PC from isotope dilution due to differences in the pool sizes of intermediate metabolites, pulse–chase experiments were performed (Figure 3). In controls, the rate of PC biosynthesis remains constant during 120 min of incubation. In the presence of 20 μM farnesol, PC biosynthesis was not inhibited during the first 30 min of incubation, but it was inhibited by 31% between 30 and 60 min and by 45% between 60 and 90 min. At the same time, labelling of CDP-choline in farnesol-treated samples was significantly increased (Figure 3, insert). No changes in labelling of choline or phosphocholine were observed (results not shown).

Since CDP-choline was the only intermediate in which farnesol caused changes in accumulation of radioactive label, we also checked the effect of farnesol on CDP-choline cellular pool size. There were no changes in CDP-choline pool size after 2 h of incubation with farnesol (1.4 $\pm$ 0.3 nmol/10^7 cells and 1.7 $\pm$ 0.1 nmol/10^7 cells in controls and treated samples respectively).

Farnesol had no effect on cell viability (Figure 2, insert) or on total choline uptake: radioactivity measured in whole cells after 2 h incubation with farnesol was (7.6 $\pm$ 0.2) $\times$ 10^5 d.p.m./10^6 cells and (7.5 $\pm$ 0.3) $\times$ 10^5 d.p.m./10^6 cells in control and farnesol-treated samples respectively.

Effect of farnesol treatment on the activity of CT and DAG concentration

To confirm that farnesol did not affect the rate-limiting enzyme of PC biosynthesis, CT activity was measured in whole cell homogenates, microsomal and cytosolic fractions. No changes in enzyme activity between control and farnesol-treated samples were found (Table 1).

Determination of the cellular level of DAG was important in the context of this work, because DAG serves as one of the

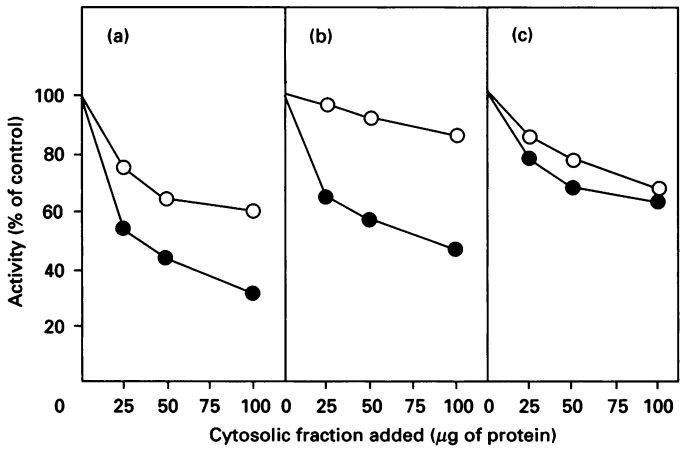
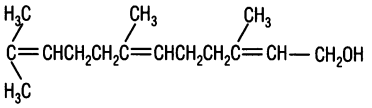
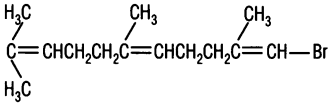
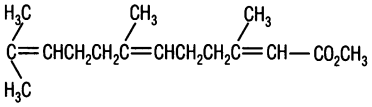
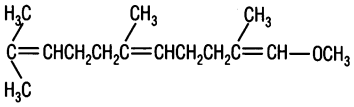
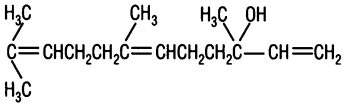
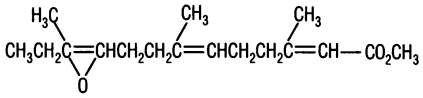
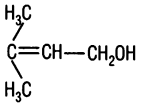
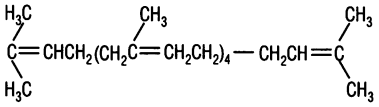
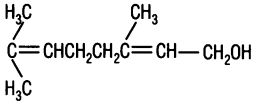
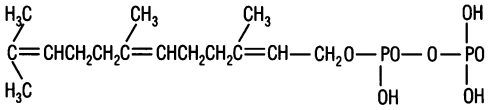


Figure 5 Effect of cytosolic fraction on CPT activity in microsomes

Cells were preincubated with 20 μM farnesol (●) or without farnesol (○) for 2 h. Microsomal and cytosolic fractions were obtained from each sample as described in the Experimental section. Part of the cytosolic fraction from each sample was left untreated (**a**). Another part was immersed in boiling water for 2 min (**b**) or dialysed against 10 mM Tris/HCl overnight (**c**). The indicated amounts of these cytosolic fractions were added to microsomes from the same sample and CPT activity was measured as described in the Experimental section. The control did not contain cytosolic fraction. Data represent percentage of control enzyme activity. Each point represents the mean of two determinations. The experiment was repeated with similar results.

Table 3 Effect of different isoprenoids on [^{3}H]choline incorporation into cellular lipids of CEM-C1 cells

Cells were incubated for 2 h with compounds indicated (20 μM each). The control contained an equivalent amount of ethanol. Samples were subjected to a 1 h pulse of labelled choline, cellular lipids were separated and their radioactivity was measured as described in the Experimental section. Data represent radioactive label incorporated, as percentage of control; S.D. in these experiments did not exceed 10% of the mean of three separate incubations.

Compound	Chemical structure	Choline incorporation (% of control)
Farnesol (*trans-trans*)		28
Farnesyl bromide		43
Farnesyl acetate		55
Farnesyl methyl ether		66
Nerolidol (mixed isomers)		69
Juvenile hormone		87
3-Methylbut-2-en-1-ol		90
Squalene		94
Geraniol		101
Farnesyl pyrophosphate		107

substrates for CPT and also can affect the activity of CT [15]. There was no significant difference in DAG concentration between control and farnesol-treated samples (Table 1).

Influence of farnesol on CPT activity

CPT activity was decreased in homogenates obtained from cells that were grown in the presence of farnesol (Table 2). But in microsomal fractions the effect was opposite, i.e. the enzyme activity was higher in farnesol-treated samples than in the controls. When farnesol was added directly to the homogenate or microsomal fractions, there was no effect on CPT activity. Preincubation of microsomal or homogenate fractions with farnesol at 37 °C for up to 1 h before enzyme assay did not change the observed effects (results not shown).

The results of analysis of microsomal CPT from control samples showed that the calculated apparent K_m value of 0.039 mM with CDP-choline as substrate was in good agreement with the values reported in the literature [8,16]. In microsomes prepared from the farnesol-treated cells this value was decreased to about half of the control, whereas the $V_{max.}$ remained unchanged (Figure 4). In order to clarify this apparent paradoxical increase in microsomal CPT activity in farnesol-treated cells, a series of mixing experiments was carried out. In these experi-

ments, increased amounts of cytosolic fractions from control and farnesol-treated cells were added to their respective microsomal preparations. The results of the assays for CPT activity (Figure 5) revealed that it was the cytosolic fraction which was responsible for decreasing the microsomal activity of CPT. That is, the difference between activities in homogenates from farnesol-treated and control cells was restored to a large extent after addition of supernatant to the microsomal fractions (Figure 5a). A similar relationship was retained when both cytosolic preparations were heated at 100 °C for 2 min (Figure 5b). However, when cytosolic fractions used in mixing experiments were dialysed against 10 mM Tris/HCl, the difference between the two preparations was abolished (Figure 5c). In other mixing experiments (results not shown), cytosol from farnesol-treated samples decreased the activity of CPT to the same approximate value regardless of whether microsomes had been obtained from control or farnesol-treated cells.

Specificity of farnesol effect on PC biosynthesis in CEM-C1 cells

To determine the specificity of the effect of farnesol on PC biosynthesis, the influence of several isoprenoid compounds on [^{3}H]choline incorporation into the lipid fraction of CEM-C1 cells was examined (Table 3). Farnesol had the most significant effect on choline incorporation. Farnesol structural analogues, namely farnesyl bromide, farnesyl acetate, farnesyl methyl ether and nerolidol, had less effect. Compounds with more significant differences, i.e. shorter (6-methylhept-5-en-2-ol, geraniol) or longer (squalene) polyprenoid chain, or with structural differences in this chain (juvenile hormone), had very little or no effect on the [^{3}H]choline incorporation into cellular lipids. No changes in cell number or viability were observed during incubation with these compounds for as long as 72 h.

DISCUSSION

In our previous paper we have shown that in lymphoid neoplastic cells (CEM-C1) toxicity of farnesol is preceded by the inhibition of choline incorporation into cellular phospholipids [5]. The results of the present study demonstrated clearly, by means of labelling of phospholipids with [^{3}H]choline, that farnesol inhibited PC biosynthesis at the level of CPT, which catalyses transfer of the phosphocholine moiety from CDP-choline to DAG. This was a surprising finding, since CPT is considered to be a non-regulatory enzyme [18]. It is noteworthy, however, that the decrease in phospholipid biosynthesis in L51784 mouse lymphoma cells treated with dexamethasone has been reported to be accompanied by a decrease of CPT activity [16].

The decrease in CPT activity in our experiments required exposure of the intact cells to farnesol, so that, when farnesol was added to cell homogenates from cells not previously treated with farnesol, the enzyme activity remained unchanged. In the experiments when cell homogenates from treated and non-treated cells were separated into cytosol and microsomal fractions, microsomal CPT from farnesol-treated cells had higher activity than that from controls. Upon re-mixing of microsomes with their respective cytosol, the original ratio of CPT activities was restored. Since cytosolic CPT activity by itself was less than 1 % of its microsomal activity, it appears that farnesol itself does not affect this enzyme activity, but is transformed into some product present in the cytosol which decreases the activity of the microsomal enzyme and may be removed during isolation of microsomes. Alternatively, farnesol may be inducing the formation of a cytosolic 'effector' that could reversibly lower the activity of CPT. This putative 'effector' was shown to be heat-stable and dialysable. Its identity as farnesol is excluded by the observation that, when added to cell homogenates or microsomes, farnesol had no effect on CPT activity even after relatively long incubation.

Our studies have also shown that inhibition of PC biosynthesis by farnesol was rather specific. Although farnesol effectively decreased PC synthesis, even its very close structural analogues had much smaller effects. Compounds with more significant structural differences, including farnesyl pyrophosphate, which was shown to be a substrate in enzymic farnesylation of proteins [4], had a very small, if any, effect on PC biosynthesis. In general, the polyprenoid chain length of three prenoid units and its original linear structure seem to be critical for affecting PC biosynthesis.

It was recently shown, that in liver of fasting hamsters PC biosynthesis is significantly decreased by action of a heat-stable dialysable cytosolic compound, which specifically inhibited CPT activity. This endogenous compound was identified as argininosuccinate [19]. Similarly to the effect of farnesol on PC biosynthesis, demonstrated in the present experiments, CT translocation between membrane and cytosolic fractions was not affected.

Since farnesol was the most active of several prenols tested and farnesyl pyrophosphate did not affect CPT activity, identification of the cytosolic 'effector' formed in response to farnesol treatment, which is currently being carried out in our laboratory, could be of potential value in defining the function of prenols in regulation of some enzymes which may not involve protein prenylation.

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