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Modulation of phosphatidylcholine biosynthesis in celery by exogenous fatty acids

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Abstract

The effects of C_{16} and C_{18} fatty acids on the synthesis of phosphatidylcholine were studied in *Apium graveolens* cell suspension cultures and postmitochondrial supernatants. When cells were exposed to exogenous oleic acid, the rate of phosphatidylcholine biosynthesis increased 1.4-fold within 5 min of the addition of the fatty acid to the culture medium. The sensitivity of microsomal CTP:cholinephosphate cytidylyltransferase (EC 2.7.7.15) to saturated and unsaturated fatty acids was monitored through the addition of unesterified fatty acids to postmitochondrial supernatants. The saturated fatty acids, palmitic and stearic, appeared to have little effect on CTP:cholinephosphate cytidylyltransferase activity, whereas exposure to oleic, linoleic and *cis*-vaccenic acids resulted in significant increases in enzyme activity. Optimal microsomal CTP:cholinephosphate cytidylyltransferase activities were achieved by the incubation of postmitochondrial supernatants with 500 μM oleate. The exogenous fatty acids were found to be incorporated into microsomal membranes in their unesterified form. Removal of unesterified fatty acids by incubation of microsomal membranes with defatted bovine serum albumin resulted in the reduction of microsomal CTP:cholinephosphate cytidylyltransferase activity; demonstrating that the enzyme requires unesterified unsaturated fatty acids.

Keywords: *Apium graveolens*; Umbelliferae; Celery; Biosynthesis; Phosphatidylcholine; CTP:cholinephosphate cytidylyltransferase

1. Introduction

Phosphatidylcholine (PC) is the major phospholipid in nonplastidial higher plant cell membranes, where it may constitute up to 40 wt% of the membrane lipid profile (Moore, 1982). The functions of PC within these membranes are multiple. For example, PC provides substrates for the synthesis of polyunsaturated fatty acids (Garces, Sarmiento, & Mancha, 1992; Williams, Williams, & Khan, 1992) and triacylglycerols (Stymne, Balfor, Jonsson, Wiberg, & Stobart, 1991) and helps to protect some plants against chilling injury (Kinney, Clarkson, & Loughman, 1987; Cheesbrough, 1989). In addition, in agreement with mammalian studies (Wieder, Haase, Geilen, & Orfanos, 1995; Vance, Houweling, Lee, & Cui, 1996; Cui et al., 1996), PC has also been shown to play a role in the regulation of plant cell division (Parkin, Goad, & Rolph, 1995).

The predominant route of PC biosynthesis in higher plants is via the CDP-base pathway (Moore, 1982). The enzymes involved in this pathway are choline kinase (EC 2.7.1.32), CTP:cholinephosphate cytidylyltransferase (EC 2.7.7.15) and phosphocholine transferase (EC 2.7.8.2). It is generally accepted that the activity of CTP:cholinephosphate cytidylyltransferase (CT) is the rate-limiting step in the production of PC (Weinhold & Feldman, 1995; Shiratori, Houweling, Zha, Tabas, 1995). As in mammalian cells, the CT enzyme of higher plant cells is topodynamically regulated. The membrane-bound enzyme has been found to be the active form in several higher plant species, such as celery and castor bean (Price-Jones & Harwood, 1983; Sauer & Robinson, 1985). However, in pea and maize, the majority of the cellular activity is located in the cytoplasm (Kinney & Moore, 1987; Parkin, Goad, & Rolph, 1993). To date, the regulation

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of CT activity has been studied in a variety of mammalian, plant and yeast species. Typical regulatory mechanisms include phosphorylation/dephosphorylation cycles, feedback inhibition involving PC and the anionic lipid content of the endoplasmic reticulum (Jamil & Vance, 1990; Cornell, 1991a; Pelech & Vance, 1984). In addition, its activity may be modulated by the presence of C_{18} unsaturated fatty acids and specific sterol molecules (Terce, Record, Tronchere, Ribbes & Chap, 1992; Parkin, Goad & Rolph, 1995). In mammalian systems, the aforementioned lipids have been shown to influence protein translocation by either enhancing the binding of the CT protein to a binding protein (Weinhold & Feldman, 1995) or by promoting the dephosphorylation of the enzyme (Shiratori et al., 1995), respectively. Nevertheless, the exact mechanisms by which these lipid molecules may regulate CT activity in higher plants remains to be elucidated. In a previous study (Parkin et al., 1995), we demonstrated the ability of oleic acid to promote CT activity in celery (*Apium graveolens*) cell suspension cultures. The work presented herein extends this study by determining the time-scale for the stimulation of CT activity by unsaturated fatty acids and by partially identifying the mechanism by which these lipid molecules may modulate CT activity.

2. Results and discussion

In order to ascertain the speed at which celery cell suspension cultures were able to take up exogenous oleic acid from the growth medium, freshly inoculated cultures were exposed to 200 μM oleic acid and the clearance of the fatty acid from the medium monitored with time (data not shown). It was observed that ca. 60% of the exogenous fatty acid had been removed within the first 24 h and 95% had been cleared after 72 h of the incubation period. Hence, it may be concluded that oleic acid had little difficulty in being transported into the celery cells.

The effects of exogenous oleic acid on PC biosynthesis in log-phase cells was monitored over a 1 h period by measuring the production of radiolabelled PC from tritiated choline. Control cultures exhibited a biosynthetic rate of 19 pmol PC min^{-1} and in cultures incubated with 200 μM oleic acid, a biosynthetic rate of 27 pmol PC min^{-1} was observed. The increase in the biosynthetic rate occurred within 5 min of the addition of oleic acid to the growth medium. This time scale for the stimulation of PC biosynthesis is not dissimilar to that demonstrated by Terce et al. for PC biosynthesis in Krebs II cells (Terce, Record, Tronchere, Ribbes, & Chap, 1992). As synthesis was promoted within minutes of the addition of oleate, it is reasonable to assume that the modulation of the activity was probably due to translocation of pre-existing enzyme protein from the cytosol to the endoplasmic reticulum, rather than de novo synthesis of active enzyme.

In order to achieve optimal CT activity, postmitochondrial supernatants prepared from log-phase cells were incubated with various concentrations of oleic acid and the resulting CT activities determined (Fig. 1). Microsomal CT activity increased in a oleic acid concentration-dependent manner, with optimal activity being observed at 500 μM oleic acid. Further increases in CT activity could not be obtained at higher oleic acid concentrations, suggesting that maximal translocation of the CT protein from the cytosol to the endoplasmic reticular membranes occurs at an oleic acid concentration of ca. 500 μM. Analysis of the lipid content of these membrane fractions revealed that the exogenous oleic acid had not become incorporated into the membrane phospholipids but simply intercalated in an unesterified form. Hence, optimal CT activities were achieved at a free fatty acid:phospholipid molar ratio of 0.06:1.0. The optimal oleate concentration necessary for maximal microsomal CT activity reported here is similar to previous studies conducted on a wide variety of mammalian cell types (Terce et al., 1992; Weinhold, Charles, Rounsifer, & Fieldman, 1991; Wang, MacDonald, & Kent, 1993).

In a further study, postmitochondrial supernatants from log-phase cultures were incubated with either 500 μM palmitic (16:0), 500 μM stearic (18:0), 500 μM oleic (18:1, Δ9), 500 μM *cis*-vaccenate (18:1, Δ11) or 500 μM linoleic (18:2, Δ9, 12) acids and the resulting CT assayed as before (Table 1). In general, treatment of the postmitochondrial supernatants with either of the saturated fatty acids had minimal effect on microsomal CT activity. With respect to the unsaturated C_{18} fatty acids, microsomal CT activity was enhanced irrespective of the degree of unsaturation or double bond position. A similar situation has been reported for the activation of purified mammalian CT in reconstituted systems comprised of different molecular species of PC (Cornell, 1991b).

It has been reported in a number of mammalian systems that oleic acid-stimulated CT activation may be reversed by the addition of BSA to microsomal membrane suspensions (Terce et al., 1992; Wang et al., 1993). In order to assess the reversibility of the oleate-induced CT activation in *A. graveolens*, postmitochondrial supernatants were incubated with 500 μM oleic acid for 30 min at 30° and microsomal membranes prepared as before. Microsomal pellets were then resuspended and the membranes incubated with defatted BSA for 15 min at 30°, following which time microsomal CT was assayed as before. Results (Table 2) demonstrated that the promotion of micro-

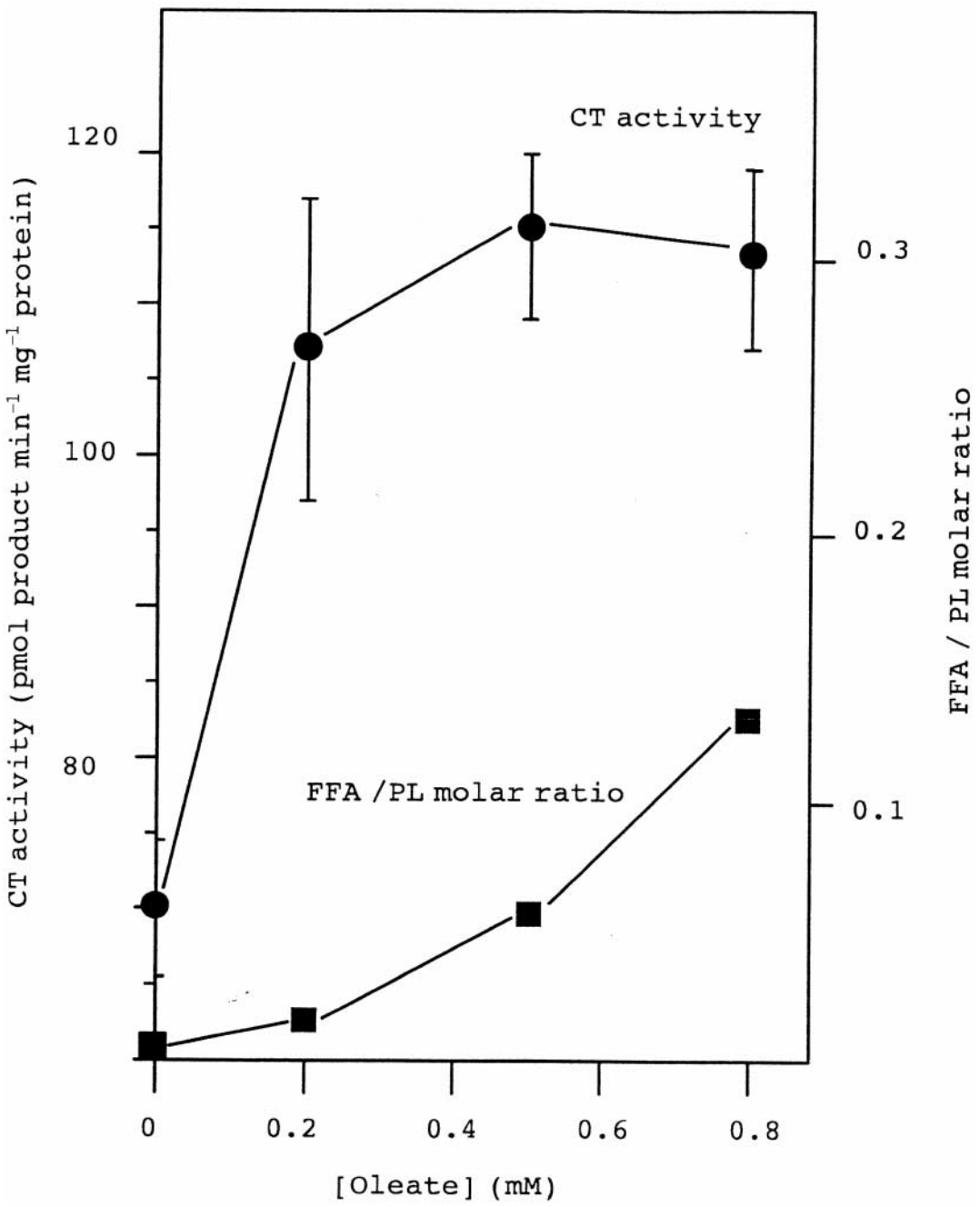

Fig. 1. Modulation of CTP:cholinephosphate cytidylyltransferase activity by exogenous unesterified oleic acid. Values represent the means of three replicates (±se).

Table 1
tEffect of unesterified fatty acid on microsomal CTP:cholinephosphate cytidylyltransferase activity

Fatty acid (500 μM)	CT activity (pmol product min^{-1} mg^{-1} protein)	Level of significance
Control	30.0 ± 0.5	
Palmitate (16:0)	36.2 ± 6.0	ns[a]
Stearate (18:0)	37.0 ± 3.5	$p < 0.05$
Oleate (18:1; Δ9)	50.0 ± 3.3	$p < 0.002$
Linoleate (18:2; Δ9, Δ12)	46.5 ± 2.1	$p < 0.002$
Vaccenate (18:1; Δ11)	50.5 ± 3.2	$p < 0.002$

Values represent the means of three replicates (±se).
[a]Not significant.

Table 2
Reversal of oleate-stimulated microsomal CTP:cholinephosphate cytidylyltransferase activity by incubation of microsomal preparations with BSA

Treatment	CT activity (pmol min^{-1} mg^{-1} protein)	Free 18:1/PL (molar ratio)
Control	120 ± 7.5	0.04
+ 500 μM 18:1	136 ± 6.0	0.12
+ 500 μM 18:1 + BSA	105 ± 6.2	0.01

Values represent the means of three replicates (±se).

somal CT activity could indeed be reversed by the presence of BSA. Indeed, the reversal of the oleic acid-stimulated CT activation was found to be concomitant with a reduction in the oleic acid:phospholipid molar ratio exhibited within the membranes.

There can be no doubt as to the importance of C_{18} unsaturated fatty acids in the regulation of CT activity. The CT enzyme present in *A. graveolens* has been shown to display similar properties to the enzyme found in mammalian systems in terms of optimal fatty acid concentration and the reversibility of the activatory process. Whether free oleic or other unsaturated C_{18} fatty acids bind firstly to the cytosolic enzyme (or to a CT binding protein (Weinhold & Feldman, 1995)) thus promoting translocation to the endoplasmic reticulum or initially intercalates with the membrane lipids thereby facilitating binding of the CT protein, remains to be elucidated.

3. Experimental

3.1. Cultures

Suspension cultures of celery (*A. graveolens* cv. New Dwarf White) were grown in Murashige and Skoog medium supplemented with kinetin and 2,4-D according to Haughan, Lenton, and Goad (1988).

3.2. Uptake of exogenous oleic acid by suspension cultures

Oleic acid dissolved in EtOH-Tergitol (2:1) was added to freshly inoculated suspension cultures to give a final concentration of 200 μM. Samples of growth media were removed, cells removed by centrifugation and the oleic acid content of the supernatant quantified by GC using pentadecanoic acid as an int. standard.

3.3. Phosphatidylcholine synthesis in whole cells

The rate of PC synthesis in log-phase cultures was determined ± oleic acid (in EtOH-Tergitol (2:1)) using [methyl^{3}H]choline according to Rolph and Goad (1991). The final concentration of oleic acid in the incubation medium was 200 μM.

3.4. Cellular fractionation

All procedures were performed at a temperature of 4°. Log-phase cells were harvested by centrifugation at 2000*g* for 10 min, washed with 10 ml homogenization buffer containing 135 mM NaCl, 55 mM *Tris* base, 1.2 mM EDTA, 1.6 mM DTT, 200 μM PMSF and 3.5 mM $MgCl_2$ (pH 7.7) and then resuspended in 25 ml of the same buffer. Membrane disruption was achieved by sonication at 18 μm for 5 min in a MSE Soniprep 150 sonicator fitted with a titanium probe. Cell debris was removed by centrifugation at 2000*g* for 10 min, following which postmitochondrial supernatants were prepared by centrifugation at 15,000*g* for 20 min. Microsomal frs were obtained by ultracentrifugation of the postmitochondrial supernatant at 119,000*g* for 1 h. The resulting pellets were resuspended in 1 ml aliquots of homogenization buffer.

3.5. Incubation of postmitochondrial supernatants with fatty acids

Postmitochondrial supernatants were incubated with a range of fatty acids dissolved in EtOH-Tergitol (2:1) for 30 min at 30°. The resulting incubations were then used for the preparation of microsomal fractions as outlined above.

3.6. Assay of CTP:cholinephosphate cytidylyltransferase

Microsomal enzyme activities were assayed using phosphoryl [methyl-^{14}C]choline, as outlined in Parkin et al. (1995).

3.7. BSA treatment of fatty acid incubated microsomal fractions

Microsomal frs prepared from fatty acid-incubated postmitochondrial supernatants were treated with

defatted BSA (final concentration, 0.33% (w/v)) for 15 min at 30°. CT assays performed as outlined above.

3.8. Extraction and analysis of membrane lipids

Microsomal lipids were treated with hot MeOH and extracted into $CHCl_3$ according to Rolphe & Goad (1991). Individual lipid classes and fatty acid compositions were determined by TLC and GC analysis (Parkin, Goad & Rolph, 1995). Quantification of membrane lipids was achieved by the inclusion of pentadecanoic acid and diheptadecanoylphosphatidylcholine in the microsomal lipid extracts.

3.9. Statistical analysis

Levels of significance were determined using the Student's *t*-test.

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