Biochem. J. (1983) **216**, 627–631
Printed in Great Britain

Hormonal regulation of phosphatidylcholine synthesis in plants

The inhibition of cytidylyltransferase activity by indol-3-ylacetic acid

Molly J. PRICE-JONES and John L. HARWOOD
Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, Wales, U.K.

(*Received 11 July 1983/Accepted 23 August 1983*)

Indol-3-ylacetic acid stimulated stem elongation within 1h of treatment of *Pisum sativum* L. cv. Feltham First stem sections. This elongation was accompanied by an increase in the endogenous level of phosphocholine and a decrease in that of CDP-choline. Measurements *in vitro* of the CDP-base pathway enzymes showed an increase in choline phosphotransferase and a decrease in cytidylyltransferase activity on hormone treatment. These results indicate that the decrease in phosphatidylcholine labelling from [^{14}C]choline that is observed on indol-3-ylacetic acid treatment of pea stem sections is caused by the decrease in cytidylyltransferase activity.

Phosphatidylcholine is the major acyl lipid of non-chloroplast membranes in higher plants (Harwood, 1980). It has been established in a number of different plant tissues that this phosphoglyceride is made primarily by the CDP-base pathway (Harwood, 1979; Mudd, 1980). The three individual enzymes of this pathway have been studied in several cases (cf. Mudd, 1980; Moore *et al.*, 1983). There have also been several reports of the effects of the plant hormone, gibberellic acid, on phosphatidylcholine metabolism. In barley, large increases have been reported in the incorporation of radioactivity from [^{32}P]orthophosphate and [^{14}C]choline into microsomal lipids, including phosphatidylcholine (Evins & Varner, 1971; Koehler & Varner, 1973). However, radiolabelling from [^{14}C]acetate or [^{3}H]glycerol was unaffected (Firn & Kende, 1974). Johnson & Kende (1971) reported that gibberellic acid increased the activity of both the cytidyltransferase and the choline phosphotransferase in barley aleurone cells, and Tanaka & Tolbert (1966) reported that gibberellic acid decreased the activity of choline kinase in spinach and squash leaves. In contrast, a recent study showed that gibberellic acid treatment caused a decrease in the total amounts of wheat aleurone tissue phosphatidylcholine by increasing its degradative rate (Mirbahar & Laidman, 1982).

We have studied the effects of indol-3-ylacetic acid on the synthesis of the major phospholipids of an auxin-responding system. We chose to use the third internode of post-germination pea seedlings, since auxin effects on such preparations have been well characterized (Evans & Ray, 1972), it is rich in phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine (Harwood, 1980; Hitchcock & Nichols, 1971) and some information is available on phospholipid synthesis in this tissue (Montague & Ray, 1977). We observed that, whereas indol-3-ylacetic acid stimulated stem elongation, it inhibited the incorporation of radioactivity from [^{14}C]choline into phosphatidylcholine within 1h of treatment (Moore *et al.*, 1983). Abscisic acid reversed these effects. No effect of indol-3-ylacetic acid on [^{14}C]ethanolamine incorporation into phosphatidylethanolamine or of [^{14}C]-acetate incorporation into lipids was observed. The amount of phosphatidylcholine on a fresh weight basis was also decreased by indol-3-ylacetic acid treatment of stem sections (Moore *et al.*, 1983). These rapid effects of auxin on phosphatidylcholine metabolism raised an obvious question: what was the metabolic site(s) of action of the hormone?

In the present paper we report results that indicate that indol-3-ylacetic acid reduces the rate of phosphatidylcholine synthesis by lowering the activity of cytidylyltransferase.

Experimental

Materials

Pea (*Pisum sativum* L. cv. Feltham First) seeds were obtained from Asmer Seeds, Leicester, U.K. [*Me*-^{14}C]Choline chloride (sp. radioactivity 2.15GBq/mmol), phospho[*Me*-^{14}C]choline (ammonium salt; sp. radioactivity 2.18GBq/mmol), cytidine 5-diphospho[*Me*-^{14}C]choline (ammonium salt; sp. radioactivity 1.92GBq/mol) and PCS scintillant were purchased from Amersham International. Indol-3-ylacetic acid, phosphocholine

chloride and CDP-choline were from Sigma (London) Chemical Co., Poole, Dorset, U.K., and Miracloth was obtained from C.P. Laboratories, Bishops Stortford, Herts., U.K. All other chemicals were of the best obtainable grades and were from Sigma or from BDH, Poole, Dorset, U.K.

Tissue preparations

Pea seeds were germinated in moist vermiculite at 20°C for 10 days in an illuminated incubator with approx. 210 (photosynthetically active wavelengths) $\mu E/m^2$ per s of illumination. Stem explants (7 mm) were excised from the third internode under 20 mM-potassium phosphate buffer, pH 6.5, weighed and pre-incubated in the buffer for 1 h at 20°C in the dark. After the pre-incubation, exposure to 10 μM-indol-3-ylacetic acid and further treatment was as described previously (Moore *et al.*, 1983).

After treatment, stem sections were homogenized in 0.32 M-sucrose/2 mM-Tris/HCl (pH 7.4) using a pestle and mortar. The homogenate was filtered through two layers of Miracloth and fractionated as described by Bolton & Harwood (1977).

Pool-size measurements

Stem explants were macerated in 15% (w/v) trichloroacetic acid, filtered through two layers of Miracloth and the filtrate was centrifuged at 10000 ***g*** for 20 min. The pellet was resuspended in fresh trichloroacetic acid and the suspension centrifuged at 10000 ***g*** for 20 min. The two supernatants were combined, the volume measured and 3.7 KBq each of [*Me*-^{14}C]choline chloride, phospho[*Me*-^{14}C]choline and cytidine 5′-diphospho[*Me*-^{14}C]choline were added. (Freeze-clamp experiments with each of the three choline-containing compounds showed that no losses occurred during maceration and centrifugation of tissue.) The supernatant was washed three times with equal volumes of diethyl ether before application of portions to preparative silica gel G thin-layer plates. The three choline-containing compounds were separated using 0.6% (w/v) NaCl/methanol/NH_3 (10:10:1, by vol.) as solvent (Choy *et al.*, 1977). Choline and phosphocholine markers were visually detected using Dragendorff reagent (Beiss, 1964) and CDP-choline marker using u.v. light. Compounds were eluted from the thin-layer plates using 0.1 M-NaOH.

Choline and phosphocholine were determined in the eluates by the method of Barak & Tuma (1981). CDP-choline was determined as phosphocholine after hydrolysis with 1 M-HCl at 100°C for 90 min. Recoveries were corrected by measuring the amount of radioactive compounds recovered.

Enzymic assays

Choline kinase (EC 2.7.1.32) activity was assayed by the method of Wharfe & Harwood (1979). Optimal assay conditions were determined using the 105000 ***g*** × 60 min supernatant and were found to be 10 mM-ATP, 10 mM-$MgCl_2$, 1 mM-dithiothreitol, 1 mM-choline chloride (containing 3.7 KBq of [*Me*-^{14}C]choline chloride), 100 mM-potassium phosphate, pH 8.5, and up to 0.3 mg of protein. Incubations were for 2 h at 25°C. The product was separated by paper chromatography using the method of Ulane *et al.* (1977), visually detected using Dragendorff spray reagent and the phosphocholine spot cut out for counting of radioactivity. Assays were carried out in triplicate and corrected for zero-time controls.

Cytidylyltransferase (EC 2.7.7.15) activity was assayed by the method of Infante & Kinsella (1978). Optimal conditions were found to be 10 mM-$MgCl_2$, 0.25 mM-ATP, 0.67 mM-CTP, 1 mM-phosphocholine (containing 3.7 KBq of phospho[*Me*-^{14}C]choline), 80 mM-Hepes/Tris, pH 7.0, and up to 0.3 mg of 105000 ***g*** × 60 min supernatant protein. Assays were for 1 h at 25°C and the products were separated by chromatography as described by Infante & Kinsella (1978). Phosphocholine was visually detected with Dragendorff spray reagent and the CDP-choline under u.v. light. The CDP-choline-containing areas were cut out for counting of radioactivity.

CDP-choline:diacylglycerol cholinephosphotransferase (EC 2.7.8.2) activity was assayed by the method of Miller & Weinhold (1981). Optimal conditions were found to be 25 mM-$MgCl_2$, 5 mM-dithiothreitol, 5 mM-EDTA, 5 mM-CDP-choline (containing 3.7 KBq of cytidine 5′-diphospho[*Me*-^{14}C]choline), 1 mM-diacylglycerol (prepared from soya-bean phosphatidylcholine by phospholipase C digestion), 0.3 mM-phosphatidylglycerol + 0.1 mg/ml final concn. of Tween 20 (all sonicated in a bath for 10 min at 65°C temperature under N_2), 100 mM-Tris/HCl, pH 8.2, and up to 0.6 mg of 18000 ***g*** × 20 min supernatant protein. Incubations were for 1 h at 25°C, and were stopped by the addition of 1 ml of chloroform/methanol (1:2, v/v). Phosphatidylcholine was extracted by the method of Garbus *et al.* (1963) and the chloroform layers sampled for radioactivity.

Alkaline phosphatase was assayed under the same conditions used for choline kinase (see above) except that the substrate was 1 mM-phosphocholine (containing 3.7 KBq of phospho[*Me*-^{14}C]choline). Products were separated by chromatography (Wharfe & Harwood, 1979) and phosphocholine and choline spots estimated for radioactivity.

Radioactive samples were counted in a scintillant consisting of PCS (Amersham-Searle)/xylene (2:1, v/v). Samples were corrected for quenching by the channels-ratio method.

Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Table 1. *Effect of indol-3-ylacetic acid treatment on stem elongation and endogenous levels of choline, phosphocholine and CDP-choline in pea stems*

Peas were germinated and stem sections treated as described in the Experimental section. Pool sizes were estimated in homogenates prepared from 40 stem sections in each case (1–1.5 g fresh weight of tissue homogenized in 15 ml of medium). All experiments were performed in triplicate as described in the Experimental section. Results are means ± S.E.M. Statistical significance was estimated by Student's *t* test for paired samples. Abbreviations used: n.s., not significant; tr, trace (<0.05 μg). Indol-3-ylacetic acid treatment was for 1 h.

Expt. no.	Sample	Stem length (mm)	Choline (μg/g fresh wt.)	Phosphocholine (μg/g fresh wt.)	CDP-choline (μg/g fresh wt.)
1	Control	7.22 ± 0.02	22.3 ± 11.2	1.1 ± 0.3	1.3 ± 0.5
	Test	7.38 ± 0.03	24.3 ± 12.7	2.3 ± 1.1	0.6 ± 0.2
2	Control	7.25 ± 0.02	11.0 ± 5.0	14.2 ± 6.5*	4.2 ± 1.7
	Test	7.47 ± 0.03	13.2 ± 3.0	6.5 ± 2.2	3.5 ± 1.9
3	Control	7.20 ± 0.03	13.2 ± 2.5	0.6 ± 0.1	0.7 ± tr
	Test	7.33 ± 0.03	11.9 ± 1.1	1.1 ± 0.1	0.3 ± 0.1
4	Control	7.21 ± 0.02	25.6 ± 2.1	1.1 ± 0.2	0.3 ± tr
	Test	7.44 ± 0.03	27.7 ± 2.2	2.0 ± 0.1	0.2 ± tr
Mean test value (% average control for each experiment)		102.7 ± 0.2 ($P < 0.001$)	102 ± 12 (n.s.)	159 ± 22 ($P < 0.05$)	78 ± 11 ($P < 0.05$)

* A single sample gave an unusually high value (27.1 μg/g fresh wt.). If this result is neglected then the average control value for Expt. 2 is 7.8 ± 1.2 μg/g fresh wt. and the mean test value (as a percentage of the average controls) becomes 171 ± 11.

Results and discussion

It is generally thought that the cytidylyltransferase is the rate-limiting step for phosphatidylcholine synthesis in mammalian tissues (Vance & Choy, 1979). However, in certain cases it is also possible that the phosphotransferase may play a regulatory role (e.g. Oldenborg & van Golde, 1977). These conclusions were reached from an examination of the relative activities of the three enzymes of the CDP-base pathway, the size of the pools of metabolic intermediates and the correlation of changes in enzyme activity with the accumulation of phosphatidylcholine (e.g. Vance & Choy, 1979).

Because of the inherent difficulty of extrapolating enzyme activities that had been assayed under optimal conditions *in vitro* to situations *in vivo*, we examined the pool sizes first. As shown in Table 1 treatment of stem sections with indol-3-ylacetic acid resulted in a decrease in the size of the CDP-choline pool and an increase in the size of the phosphocholine pool. This result suggested that the cytidylyltransferase step was inhibited and, possibly, also that the phosphotransferase step was stimulated.

We next examined the three enzymes concerned in the CDP-base pathway. Choline kinase is a soluble enzyme in plant tissues (Wharfe & Harwood, 1979) and cholinephosphotransferase activity is located in microsomal fractions (Lord, 1975; Moore *et al.*, 1983). These enzymes were assayed in the 105000 *g* supernatant and the postmitochondrial supernatant respectively. We tested the cytidylyltransferase of pea stems and, as in the case of onion stems (Morre *et al.*, 1970), we found that the enzyme was found in both the microsomal (6%) and the soluble fractions (75% of recovered activity) (see the Experimental section). Accordingly cytidylyltransferase activity was assayed in the 105000 *g* supernatant. Optimal conditions were found for each enzyme, which was assayed as described in the Experimental section. However, choline kinase activity was unexpectedly low and it was observed that the reaction was non-linear with time. It was found that pea stems contained an extremely active alkaline phosphatase that hydrolysed the phosphocholine product. By analogy with the bacterial enzyme (Torriani, 1960) we attempted to inhibit activity with P_i. However, even at 100 mM-phosphate concentrations we were only able to achieve a 10% reduction in the rate of phosphocholine hydrolysis. The values given in Table 2 for choline kinase are therefore likely to be underestimations of the maximal rates. However, it will be seen that, whereas indol-3-ylacetic acid treatment of pea stem sections decreased activity of cytidylyltransferase, it did not affect choline kinase activity measured under steady-state conditions. In addition, indol-3-ylacetic acid treatment had no effect on the the time course of the choline kinase reaction or on the extrapolated initial reaction rates (results not shown). Hormone treatment of pea stems did, however, result in an increased choline phosphotransferase activity (Table 2).

The rapid effect of indol-3-ylacetic acid treatment of pea stems in inhibiting the incorporation of [^{14}C]choline into phosphatidylcholine appears to be due to an inhibition of cytidylyltransferase activity.

Table 2. *Effect of indol-3-ylacetic acid treatment on stem elongation and on the activities of enzymes involved in phosphatidylcholine synthesis in pea stems*

Peas were germinated and grown and stem explants prepared and treated as described in the Experimental section; 40 explants were used for stem length measurements in each experiment (about 1g fresh weight of tissue was homogenized in 10ml of medium). Enzyme activities were measured in the 105000g × 60min supernatant (choline kinase and cytidylyltransferase) or the 18000g × 20min supernatant (cholinephosphotransferase) as described in the Experimental section. Triplicate determination for fractions prepared from 40 stem sections was used in each case. Results are expressed as means ± S.E.M. Significance was estimated by Student's t test for paired samples. Abbreviation used: n.s., not significant.

Expt. no.	Sample	Stem length (mm)	Choline kinase (nmol/min per mg of protein)	Stem length (mm)	Cytidylyltransferase (nmol/min per mg of protein)	Stem length (mm)	Cholinephosphotransferase (nmol/min per mg of protein)
1	Control	7.22 ± 0.02	0.06 ± 0.01	7.14 ± 0.02	6.53 ± 0.44	7.13 ± 0.01	2.47 ± 0.11
	Test	7.43 ± 0.03	0.08 ± 0.01	7.30 ± 0.03	6.27 ± 0.54	7.24 ± 0.02	2.81 ± 0.13
2	Control	7.17 ± 0.02	0.10	7.25 ± 0.03	11.33 ± 1.26	7.15 ± 0.02	0.07 ± 0.06
	Test	7.34 ± 0.03	0.04	7.44 ± 0.05	9.20 ± 0.51	7.41 ± 0.03	0.56 ± 0.39
3	Control	7.21 ± 0.02	0.05 ± 0.01	7.18 ± 0.03	12.95 ± 0.60	7.20 ± 0.02	0.26 ± 0.23
	Test	7.46 ± 0.03	0.06 ± 0.02	7.43 ± 0.04	9.70 ± 0.30	7.52 ± 0.05	2.05 ± 0.26
4	Control			7.22 ± 0.02	9.23 ± 1.54		
	Test			7.43 ± 0.03	0.04 ± 0.01		
Mean test values (% average control for each experiment)		102.9 ± 0.3 (P < 0.001)	105.4 ± 13.1 (n.s.)	102.3 ± 0.3 (P < 0.001)	62.3 ± 8.3 (P < 0.01)	103.0 ± 0.3 (P < 0.001)	280.2 ± 14.5 (P < 0.05)

Thus the pool size of phosphocholine was found to increase, whereas that of CDP-choline decreased. In addition, measurements of cytidylyltransferase activity showed a significant decrease in hormone-treated tissues. It is not known at present whether this change in cytidylyltransferase activity is due to protein turnover or deactivation. The possible effect of hormones in raising intracellular [phosphatidylglycerol] (Khosla *et al.*, 1980) and so stimulating cytidylyltransferase activity (cf. Feldman *et al.*, 1978) does not seem to be important, since the pea enzyme is not stimulated by the phospholipid (results not shown). However, the pea system does appear to be similar to mammalian tissues in that the cytidylyltransferase seems to be the key enzyme in regulating choline incorporation into phosphatidylcholine by the CDP-base pathway.

The financial support of the Agricultural Research Council is gratefully acknowledged.

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