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Gibberellin modulation of phosphatidyl-choline turnover in wheat aleurone tissue

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Abstract. Phosphatidyl choline (PC) is synthesised in wheat (*Triticum aestivum* L. cv. Flanders) aleurone tissue during early germination when new endomembranes are being formed. Although gibberellic acid does not ostensibly affect PC levels, it inhibits the incorporation of choline and differentially and specifically modulates the turnover of the N-methyl and methylene carbons of the choline headgroup of PC. Gibberellic acid has no effect on turnover of the phosphate moiety of either PC or the other major phosphatides. The possible biological importance of the findings is discussed.

Key words: Aleurone – Germination (seed) – Gibberellin and phospholipid turnover – Membranes – Phospholipid – *Triticum* (gibberellin and phospholipids).

Introduction

In the cereal aleurone tissue during early germination and prior to the appearance of gibberellin (GA)-induced α-amylase activity, there is a rapid net synthesis of phospholipid and formation of endoplasmic reticulum (ER) (see Laidman 1982). Early studies on barley indicated that the formation of ER cisternae was induced by GA (Jones 1969; Vigil and Ruddat 1973) and GA action led to an enhanced incorporation of radiolabelled choline and orthophosphate into phospholipids (Evins and Varner 1971; Koehler and Varner, 1973). Experiments on wheat (Laidman et al., 1974) directly contradicted these findings however. Further experiments on barley also cast some doubts, since GA did not affect the incorporation of radiolabelled glycerol or acetate into the phospholipids and it did not affect gross phospholipid levels in the tissue (Koehler and Varner 1973; Firn and Kende 1974). Subsequently, the initiation of ER formation in barley was also shown to be independent of GA (Jones 1980), thereby agreeing with the results obtained for wheat. Several of the early reports on barley have thus been discounted, but the possibility remains that GA regulates the turnover of phospholipids, especially phosphatidyl choline (PC), during the lag period leading to α-amylase production. We have therefore made a more detailed study of the synthesis and turnover of this phosphatide in wheat aleurone tissue using radiolabelled precursors and more stringent experimental conditions.

Abbreviations: ER = endoplasmic reticulum; GA = gibberellin; GA_3 = gibberellic acid; PA = phosphatidic acid; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PG = phosphatidyl glycerol; PI = phosphatidyl inositol; $t_{1/2}$ = half-life

Material and methods

Preparation and incubation of plant material. Soft-grained winter wheat (*Triticum aestivum* L. cv. Flanders, R.H.M. Research and Engineering, High Wycombe, Bucks., UK) was used in batches of 30 grains. The embryo-containing end of each grain was removed with a transverse cut, and the embryoectomized grains were sterilized in dilute NaOCl (0.15% available chlorine) under reduced pressure (Mirbahar and Laidman 1982). All subsequent treatments were carried out under aseptic conditions. The embryoectomized grains were incubated in the dark at 25° C with distilled water ± 1 μM gibberellic acid (GA_3). After incubation for up to 72 h, the embryoectomized grains were dissected to obtain their aleurone layers.

Radiolabelling of aleurone tissue. For precursor uptake and incorporation measurements, isolated aleurone layers from 30 embryoectomized grains were radiolabelled for 30 min at 25° C in 10 ml labelling medium contained in a 25-ml conical flask. The labelling medium contained 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-maleate buffer, 50 $\mu g \cdot ml^{-1}$ chloramphenicol and either 185 kBq [CH_3-^{14}C]choline (2.1 $GBq \cdot mmol^{-1}$), 185 kBq[1,2-^{14}C]choline (270 $MBq \cdot mmol^{-1}$) or 3.7 MBq ^{32}P-orthophosphate (carrier-free, Amersham International, Amersham, Bucks., UK). Cold-carrier (unlabelled) choline chloride or K-orthophosphate, pH 6.7, was added as specified in the results section. The flask and contents

were incubated at 50 oscillations·min^{-1} in a reciprocating water bath. At the end of the incubation, the tissue was removed from the medium, washed several times in distilled water, incubated for 5 min in 40 mM unlabelled choline chloride or 1 mM K-orthophosphate, pH 6.7, and washed again in distilled water.

For measurements of phospholipid turnover, aleurone layers were pulse-labelled for 10 min ([^{14}C]choline) or 30 min (^{32}P-orthophosphate) using the labelling medium described above, but with the cold carrier excluded. At the end of the pulse, the tissue was washed in distilled water, and incubated for 5 min in 100 ml 1 mM unlabelled choline chloride or 1 mM unlabelled K-orthophosphate, pH 6.7. The tissue was then washed again in distilled water and transferred to a 25-ml conical flask containing 10 ml chase medium. The chase medium consisted of 50 mM Tris-maleate buffer, pH 6.7, 50 µg·ml^{-1} chloramphenicol, and either 1 mM unlabelled choline chloride or 1 mM K-orthophosphate, pH 6.7. The tissue was incubated for up to 72 h at 25° C in this medium. At the end of the chase incubation the tissue was removed from the medium and washed several times with distilled water.

Extraction and analysis of phospholipid. The washed aleurone layers were damp-dried and extracted with hot water-saturated butan-1-ol. An aliquot of the extract was taken to measure the uptake of radioactivity into the tissue. The crude lipid extract was then purified by partition chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) and total lipid phosphorus was determined on aliquots taken from this purified extract. The purified extract was fractionated into neutral lipids, glycolipids and phospholipids by silicic-acid column chromatography and the phospholipid fraction was further separated into individual phosphatide classes by two-dimensional thin-layer chromatography on silica gel G (BDH, Poole, Dorset, UK). This provided fractions for the determination of the individual phosphatides. These methods have been described in detail previously (Colborne and Laidman 1975). Phospholipids were determined as inorganic orthophosphate and radioactivity in the phospholipids was determined by scintillation spectrometry (Varty and Laidman 1976). Since radiolabelled choline incorporated into the lipid fraction is located specifically in PC, radioactivity in this compound could be determined using the purified extract from Sephadex G-25 chromatography. ^{32}P-radioactivity in total phospholipids was determined using the same fraction, while ^{32}P-radioactivity in individual phosphatides was determined in the fractions from thin-layer chromatography.

Results

The results in Figs. 1 and 2 are from one batch of seeds, while the results in Figs. 3 and 4 and in the tables are from another batch.

Levels of PC. Gibberellic-acid-induced α-amylase activity was first detected in embryoectomized seeds after 24 h of incubation (data not presented). During this 24-h lag period, the PC level in the aleurone tissue increased about two fold in both the control and GA_3-treated tissue (Fig. 1). After 24 h, in the control tissue the level continued to increase slowly, while the level in the GA_3-treated tissue remained constant up to 48 h and then decreased.

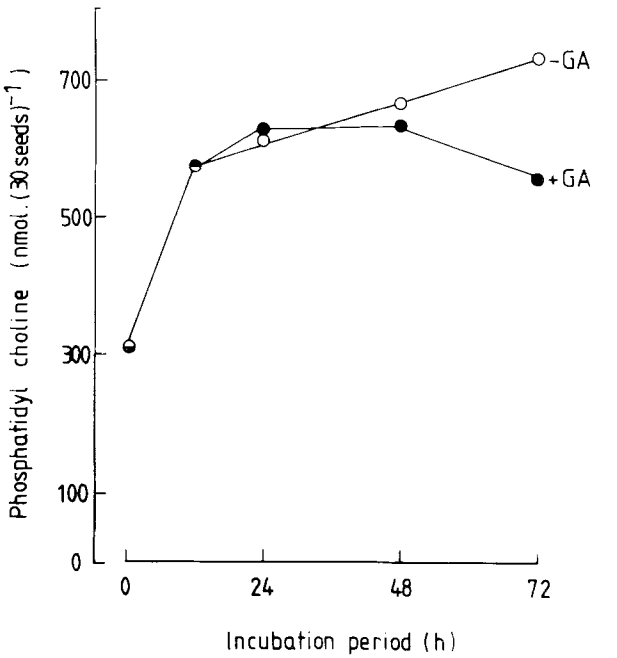

Fig. 1. Phosphatidyl choline levels in aleurone tissue of incubated embryoectomized wheat grains. o—o, −GA (control); •—•, +GA

Incorporation of precursors. The uptake of radiolabelled precursors into the tissue and their incorporation into PC were determined using aleurone tissue isolated from embryoectomized seeds which had been incubated for up to 72 h either in the absence or in the presence of GA_3.

Uptake and incorporation of [CH_3-^{14}C]choline were greatest at pH 6.7 and both the uptake and the incorporation were saturated in the presence of 40 mM unlabelled carrier. These incubation conditions were therefore used in all experiments. Under these conditions, GA_3 had no effect on the uptake of radiolabelled choline into the tissue, and attention was therefore focussed on its incorporation into PC. Incorporation was greatest in freshly imbibed tissue (Fig. 2). Incorporation then declined progressively in the control tissue. In the GA_3-treated tissue, incorporation was reduced below the control values between 6 and 20 h with a minimum incorporation at 12–14 h. Incorporation then increased, and between 24 and 72 h it was slightly more than that in the control. At 12–14 h, when the difference between control and GA-treated tissue was greatest, the incorporations of [CH_3-^{14}C]choline and [1,2-^{14}C]choline into PC were the same (Table 1). This implies that the choline molecule is incorporated as a whole during short incubations (30 min) involving saturating conditions. In contrast to its effect on incorpora-

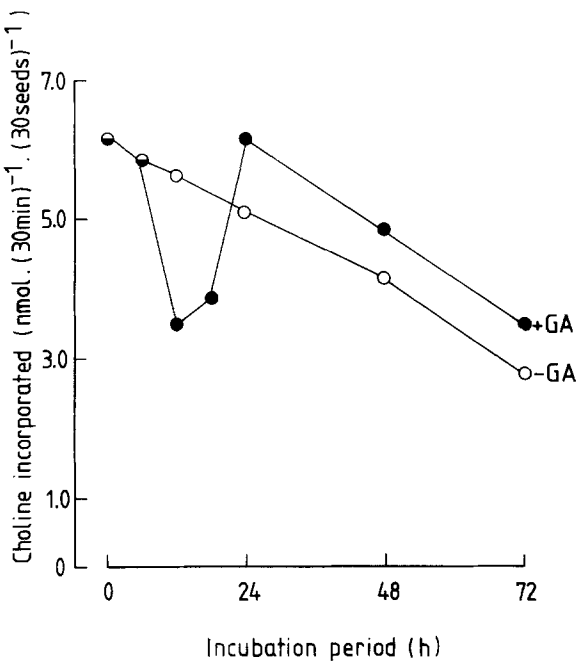

Fig. 2. Incorporation of [CH_3-^{14}C]choline into phosphatidyl choline in aleurone tissue of incubated embryoectomized wheat grains. o—o, −GA (control); •—•, +GA

tion, GA_3 had no appreciable affect on the uptake of radiolabelled choline into the tissue. The results in Figs. 1 and 2 were checked using three different grain batches from the same cultivar. The data for PC levels (Fig. 1) were very reproducible. The data for (CH_3-^{14}C)choline incorporation (Fig. 2) were qualitatively similar for the different batches. In particular, the strong inhibitory effect of GA was always observed at 14 h of incubation. The batches gave quantitatively different incorporation results, however, due in part at least to different choline-uptake kinetics. The results in Fig. 2 and Table 1 are therefore not strictly comparable, since they are derived from different seed batches.

Incorporation of radiolabelled orthophosphate into total phospholipids was not completely saturated even at 50 mM carrier orthophosphate. At this concentration and using tissue from endosperms incubated for 14 h, incorporation was only 2.2 nmol/30 seeds compared with 142 nmol/30 seeds for choline. Treatment with GA_3 had no appreciable effect on the value. Because of the low incorporation and the failure to saturate uptake and incorporation, the effect of GA_3 on orthophosphate incorporation into individual phosphatides was studied in the absence of carrier. Under this condition PC incorporated most radioactivity (Table 2) and hormone treatment had no effect on incorporation into any of the phosphatides.

Table 1. Uptake of radiolabelled choline into aleurone tissue and its incorporation into phosphatidyl choline. Both uptake and incorporation were measured in the presence of 40 mM choline chloride. Each value is the average from two experiments which agreed within 10%. nd = not determined

	GA	Uptake (nmol ·(30 grains)$^{-1}$)	Incorporation (nmol ·(30 grains)$^{-1}$)
[CH_3-^{14}C]choline	−	1980	142
	+	2220	56
[1,2-^{14}C]choline	−	nd	149
	+	nd	60

Table 2. Incorporation of ^{32}P-orthophosphate into phospholipids. The incorporation was measured in the absence of carrier orthophosphate. Each value is the average from two experiments which agreed within 10%

	Incorporation	
	−GA	+GA
Total phospholipids (dpm/30 grains)	4890	5130
Individual phosphatides (% of total)		
PC	51	52
PE	10	10
PG	8.9	8.6
PI	6.2	6.8
PA	3.2	3.5

Headgroup turnover. Turnover was estimated using [CH_3-^{14}C]choline, [1,2-^{14}C]choline and ^{32}P-orthophosphate as precursors. Labelling the PC with [CH_3-^{14}C]choline allows the turnover of the N-methyl groups of the choline moiety to be estimated, while [1,2-^{14}C]choline permits the turnover of the methylene carbons (the backbone) of the choline moiety to be measured. Using ^{32}P-orthophosphate, the turnover of the phosphate moiety of several phosphatides including PC can be measured. These experiments were carried out on aleurone tissue from embryoectomized grains which had been incubated for 12 h ± GA_3.

After pulse-labelling for 10 min with [CH_3-^{14}C]choline, radioactivity continued to accumulate in PC during the first 2 h of the subsequent chase incubation. Afterwards, radioactivity in PC decreased exponentially and semi-logarithmic plots of the data gave reasonably straight lines at least up to 12 h (Fig. 3A). The slopes of these lines corresponded to half-lives ($t_{1/2}$) of 6 h for the control tissue and 33 h for the GA_3-treated tissue. Using

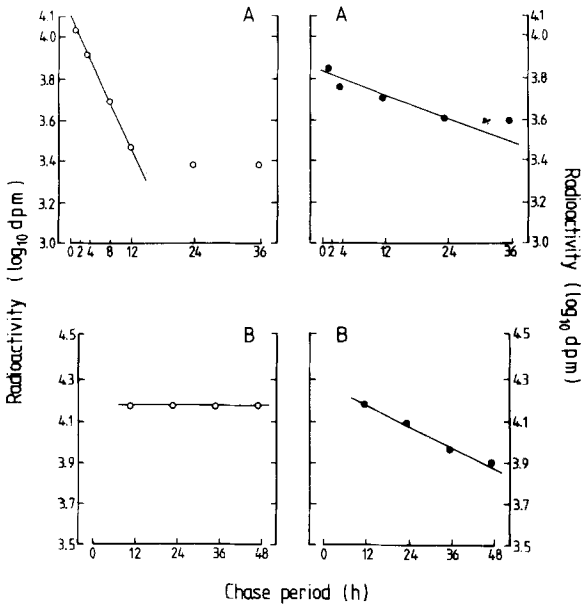

Fig. 3A, B. Turnover of phosphatidyl choline labelled with either [CH_3-^{14}C]choline (**A**) or [1,2-^{14}C]choline (**B**). o—o, −GA (control); ●—●, +GA. See *Material and methods* for experimental details

Table 3. Turnover of radiolabelled phospholipids. The half-life values are derived from the semi-logarithmic plots in Fig. 4. The values in parenthesis are the Pearson correlation coefficients for the semi-logarithmic plots

Precursor	Phosphatide labelled	Half-life	
		−GA	+GA
[CH_3-^{14}C]choline	PC	6	33
[1,2-^{14}C]choline	PC	>100	34
^{32}P-orthophosphate	PC	53 (0.96)	60 (0.99)
	PE	23 (0.94)	27 (0.88)
	PG	42 (0.93)	77 (0.40)
	PI	58 (0.91)	62 (0.85)
	PA	64 (0.97)	42 (0.78)

[1,2-^{14}C]choline as the precursor in otherwise identical experimental conditions, the results shown in Fig. 3B were obtained. The slopes of these semi-logarithmic plots gave $t_{1/2}$ values of >100 h for the control and 34 h for the GA_3-treated tissue. The values for the two radiolabelled cholines are summarised in Table 3.

In pulse-chase experiments using ^{32}P-orthophosphate, most (60–70%) of the radioactivity was incorporated into PC. Semi-logarithmic plots of the radioactivity decay data for this phosphatide gave straight lines (Fig. 4) with gradients corresponding to $t_{1/2}$ values of 53 h and 60 h for the control and GA_3-treated tissue, respectively (Table 3). The difference between these figures is probably not statistically significant.

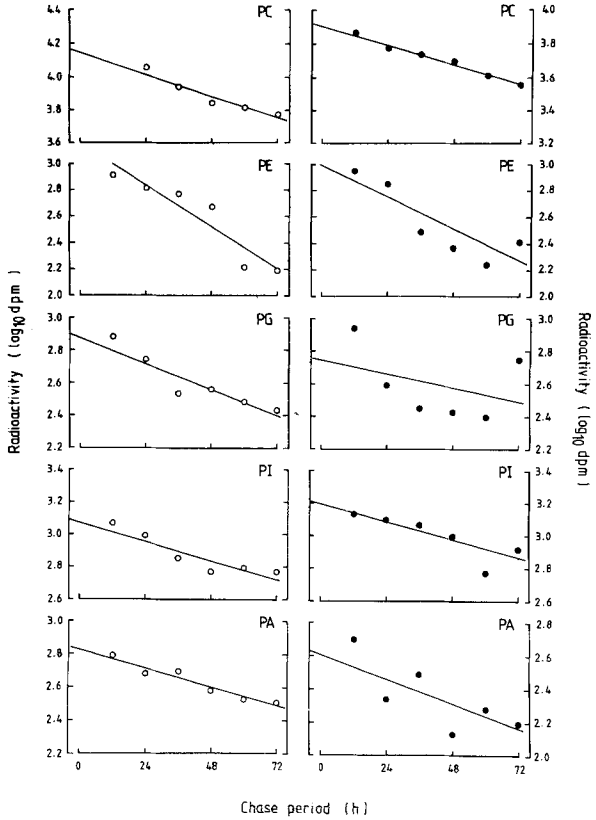

Fig. 4. Turnover of phosphatides labelled with ^{32}P-orthophosphate. o—o, −GA (control); ●—●, +GA. *Material and methods* for experimental details

Smaller amounts of radioactivity were incorporated from ^{32}P-orthophosphate into phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), phosphatidyl inositol (PI) and phosphatidic acid (PA). The amounts were sufficient, however, to allow rough estimates of their half-lives to be made, and these results are included in Fig. 4 and Table 3. Two conclusions can be drawn from the results. Firstly, the $t_{1/2}$ values extended from about 21 h for PE to about 64 h in the case of PA. Secondly, the data contain no evidence for a GA effect on turnover in any of the phosphatides. (The values for PG and PA in the GA_3-treated tissue are not reliable, since the correlation coefficients for their plots are low compared with those for the other phosphatides; see Table 3.)

Discussion

The finding that PC levels in the aleurone tissue increase more than two fold during the first 24 h

of germination complements our earlier observation (Varty and Laidman 1976) that total phospholipid levels increase similarly during this period. The finding that GA_3 has no detectable effect on PC levels also reflects the absence of a GA effect on total phospholipid levels. It is therefore unexpected that GA_3 strongly inhibits the incorporation of choline into PC. This finding, made under conditions where the uptake of choline was saturated, is at variance with the earlier data of Varty and Laidman (1976) and completely contradicts the findings of Evins and Varner (1971). Both of the latter studies were, however, carried out under non-saturating conditions in which the uptake of choline into the tissue was presumably the rate-limiting step, and their findings must now be considered wrong.

We are unable to offer a confirmed explanation for the transient inhibition of choline incorporation by GA_3. It is unlikely to be the result of recycling of radiolabelled choline from PC breakdown, since GA_3 affects choline turnover in PC differently from its incorporation into PC (compare Tables 1 and 3). It might be the consequence of an increase in the choline pool size resulting from the GA_3 action. Our attempts to check this possibility have so far been unsuccessful because of the low levels of choline present (data not shown), and further work is needed.

The rate at which radiolabelled choline was incorporated into PC during early germination in our present experiments is sufficient to account for the observed rate of increase in PC levels. Thus, at 14 h the PC accumulation determined from Fig. 1 is about 13 $nmol \cdot h^{-1}$, which compares with a choline incorporation of 11 $nmol \cdot h^{-1}$ (from Fig. 2). The rate of choline incorporation in Table 1 (146 $nmol \cdot h^{-1}$) is, however, in excess of that needed for the observed PC accumulation. As mentioned above, the data in Fig. 2 and Table 1 were obtained from different seed lots which had different choline-uptake kinetics.

Following the incorporation of the intact choline molecule into PC, turnover of the choline moiety clearly proceeds at two levels involving turnover of the N-methyl groups on the one hand and the whole choline group on the other. These activities are regulated in different ways by GA_3. Thus, turnover of the choline moiety in the control tissue is confined to the N-methyl groups, turnover of the choline group per se being very slow ($t_{1/2} > 100$ h). The effect of incubating the tissue with GA_3 is to stop the independent turnover of the N-methyl groups and to initiate the turnover of the whole choline moiety ($t_{1/2} = 34$ h). This response to GA_3 is specific to the choline moiety of PC, there being no effect of the hormone on turnover of the phosphate moiety.

The half-life values for the phosphate groups in the various phosphatides are in general agreement with values reported for other plant tissues (Mazliak 1980). Despite this general consensus, turnover of the phosphate group of PC in the control tissue ($t_{1/2} = 53$ h) is surprisingly faster than that of the choline moiety ($t_{1/2} > 100$ h). This apparent anomaly may be the consequence of the different chase conditions which were necessarily used to estimate the turnovers of the two groups. Any error would, however, be expected to overestimate the phosphate half-life value compared with the value for choline, since the concentration of free choline in aleurone tissue is very low and smaller than that of orthophosphate (Vakharia 1986). The apparent anomaly remains therefore.

Two hypotheses can be proposed to explain the biological importance of the GA_3-modulated PC turnover. In the first hypothesis, modulation of the choline-moiety turnover is an early event in a signal transduction sequence which starts with gibberellin-receptor binding and ends with the induction of protein (α-amylase) synthesis and secretion. This hypothesis is supported by a number of points. The GA_3 modulation of PC-headgroup turnover occurs at the correct time during the lag period leading to GA_3-induced protein (α-amylase) production. Regulation of membrane phospholipid methylation by cell-surface receptors, involving both N-methylation of PE and the incorporation of choline into PC, has been proposed for several animal tissues (Mato and Alemany 1983). Our data for phosphate-group turnover in the other phosphatides indicates that GA_3 modulation of turnover does not occur at this time, in contrast to the GA_3-stimulated turnover of phosphatides which occurs later during germination when α-amylase is being actively secreted from the tissue (Mirbahar and Laidman 1982). The GA signal transduction mechanism in the aleurone tissue may therefore involve the turnover of the PC headgroup rather than that of PI. In the second hypothesis, turnover of the PC headgroups is part of the mechanism for membrane assembly, especially the conversion of pre-ER to ER. The pre-ER in cereal aleurone tissue is formed during early germination (up to 14 h) when it is observed in close association with the aleurone grain-oleosome complex (Jelsema et al. 1980). This is followed by a decrease in the amount of pre-ER and an increase in the amount of ER lamellae, indicating a precursor-product relationship. The present hypothesis sub-

mits that GA regulates PC turnover associated with the membrane-assembly mechanism. It is noteworthy that the PC synthesis and turnover which we have studied takes place in the aleurone grain-oleosome fraction of the cell (Wilkinson et al. 1984; Brearley 1986). Further studies are needed to resolve the two hypotheses.

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