Are Polyphosphoinositides Involved in Signal Transduction of Elicitor-Induced Phytoalexin Synthesis in Cultured Plant Cells?

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Polyphosphoinositides, Elicitors, Parsley *(Petroselinum crispum)* Cells, Soybean *(Glycine max)* Cells, Turnover

The phospholipids of cultured parsley and soybean cells were labelled with myo-[2-^{3}H]inositol, [2-^{3}H]glycerol or [^{32}P]orthophosphate. By one- and two-dimensional chromatographic comparison of the labelled phospholipids with reference substances, the presence of 1-(3-sn-phosphatidyl)-D-myo-inositol 4-phosphate and 1-(3-sn-phosphatidyl)-D-myo-inositol 4,5-bisphosphate was demonstrated in these cultures. These results were corroborated by analysis of the deacylation products. Cells were labelled with either myo-[2-^{3}H]inositol, [2-^{3}H]glycerol or [^{32}P]orthophosphate and subsequently challenged with elicitor for various lengths of time. Radioactivity in individual phosphoinositides from these cells was determined. No significant influence of elicitor-challenge of either soybean or parsley cells on incorporation of ^{3}H or ^{32}P into polyphosphoinositides was found between 0.5 and 20 min after elicitor addition.

Introduction

Phytoalexins are low molecular weight, antimicrobial compounds that accumulate in plants at infection sites [1]. Their accumulation can be induced *in vitro* by treating plants or cultured plant cells with abiotic or biotic molecules called elicitors [2, 3]. In cell cultures of soybean *(Glycine max),* French bean *(Phaseolus vulgaris),* and parsley *(Petroselinum crispum)* elicitor treatment leads to increases in amount and activity of mRNAs encoding enzymes of phytoalexin biosynthesis [4–6]. Furthermore, the accumulation of these mRNAs in parsley and bean cell cultures was shown to result from transient increases in their transcriptional rates [7–9]. It can, therefore, be postulated that specific genes involved in phytoalexin synthesis and in other possible defense reactions [3] become activated upon the elicitor-plant cell interaction.

The effects induced in parsley cells by an elicitor from *Alternaria carthami* can be fully reversed provided that the cells are freed of elicitor within 20 minutes following its addition [10]. Furthermore, a high affinity binding site for a glucan elicitor from *Phythophthora megasperma* f. sp. *glycinea* has recently been detected in a plasma membrane-enriched fraction of soybean tissues (W. Schmidt and J. Ebel, personal communication). The binding affinity of this elicitor and that of other elicitors correlate with their relative efficiency in inducing glyceollin (soybean phytoalexin) accumulation.

From the results described above it can be assumed that, after binding of elicitor to the plasma membrane, a signal is transmitted to the nucleus. The nature of this signal is so far unknown. Rapid changes in the intracellular levels of inorganic phosphate are induced in parsley cells upon addition of *Ac* elicitor [11]. In soybean hypocotyls, the presence of cAMP could be shown by a radioimmunoassay [12]. However, no correlation was observed between infection with race 1 of *Pmg* (incompatible with soybean) and soybean cAMP levels, nor was cAMP found in cultured soybean cells [12]. It therefore appears unlikely that cAMP is involved in such a signal transmission.

In vertebrate cells, D-myo-inositol, 1,4,5-trisphosphate (Ins-P_3) was shown to function as a messenger in cellular signal transduction [13]. Phosphatidylinositol 4,5-bisphosphate is hydrolyzed to diacylglycerol and Ins-P_3 in response to external stimuli. Diacylglycerol then activates protein kinase C, whereas Ins-P_3 mediates the release of calcium from

Abbreviations: PtdIns, 1-(3-sn-phosphatidyl)-D-myo-inositol; PtdIns-4P, 1-(3-sn-phosphatidyl)-D-myo-inositol 4-phosphate; PtdIns-4,5P_2, 1-(3-sn-phosphatidyl)-D-myo-inositol 4,5-bisphosphate; Ins-P, D-myo-inositol monophosphate; Ins-P_2, D-myo-inositol bisphosphate; Ins-P_3, D-myo-inositol trisphosphate; *Pmg, Phytophthora megasperma* f. sp. *glycinea; Ac, Alternaria carthami* Chowdhury.

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intracellular storage sites [13]. Ebel *et al.* [14] reported recently that Ca^{2+} ions stimulate the effect of *Pmg* elicitor on glyceollin accumulation and phenylalanine ammonia-lyase and chalcone synthase activities in cultured soybean cells. These results suggest that changes in intracellular Ca^{2+} pools could possibly be involved in signal transmission. Exact determination of the intracellular Ca^{2+} concentration has, however, not yet been possible. We were interested in finding out whether elicitor-stimulated polyphosphoinositide metabolism might play a role in elicitor action on plant cells. Except for a preliminary comment concerning radish [15], it was unknown at the beginning of our investigation whether polyphosphoinositides occur at all in higher plants. Here we report on the presence of such compounds in cultured cells of parsley and soybean and on possible effects of *Pmg* elicitor and *Ac* elicitor on the turnover of these compounds.

Materials and Methods

Cell cultures

Cell suspension cultures of parsley (*Petroselinum crispum* Nym.) and soybean (*Glycine max* [L.] Merill cv. Harosoy 63) were grown in the dark as described previously [16, 17].

Materials

Myo-[2-^{3}H]inositol (577 TBq/mol), [2-^{3}H]glycerol (37 TBq/mol) and [^{32}P]orthophosphate were purchased from Amersham-Buchler, Braunschweig. A polyphosphoinositide mixture (PtdIns-4P and PtdIns-4,5P_2) was from Sigma, Steinheim. Tetrabutylammonium hydrogen sulfate was obtained from Serva, Heidelberg, and the anion exchange resin AG 1×2 (200–400 mesh) was purchased from BioRad Laboratories, München. All other chemicals were of analytical grade.

Thin layer chromatography

Silica 60 thin-layer plates (Merck, Darmstadt) were impregnated with oxalic acid by dipping them into a solution of 1% potassium oxalate in methanol/water 60:40 (v/v) [18]. The plates were subsequently airdried and activated for 2 h at 70 °C. The following solvent systems were used (by volume): (1) trichloromethane/methanol/4 N ammonia 9:7:2 (first dimension); (2) trichloromethane/acetone/methanol/acetic acid/water 45:15:13:7 (second dimension). Sufficient separation of the phospholipids was achieved by developing the chromatograms one-dimensionally for 2.5–3 h. Phospholipids were located either by exposing the plates to iodine vapor [19] or by spraying them with a molybdenum spray [20].

Phosphate determination

The phosphate content of the isolated phospholipids was determined according to Ames and Dubin [21].

Incubation of cells with radioactive precursors and extraction of phospholipids

Phospholipids were extracted from the cells by a modification of the procedure described by Grove *et al.* [22]. Cell suspension cultures of parsley (6 day-old) or soybean (10 h and 5 day-old, respectively) were incubated in the dark with shaking for 24 h with 0.5 μCi/ml culture of either [2-^{3}H]inositol, [2-^{3}H]glycerol or [^{32}P]orthophosphate. The cells were subsequently harvested by vacuum filtration and placed immediately into test tubes filled with an ice-cold mixture of trichloromethane/methanol 1:1 (v/v) containing 10 mM tetrabutylammonium hydrogen sulfate [22] (4 ml/about 1 g of cells). The suspension was stirred for 15 min and sedimented by centrifugation (5 min, $3000 \times g$). Extraction of the cells was repeated twice, and the combined supernatants were concentrated in a rotary evaporator under N_2 to about half their volume, after which trichloromethane and 0.1 N HCl (2 ml each) was added. The aqueous phase was discarded after extraction, and the trichloromethane phase was washed three times with 0.1 N HCl (2 ml each) which had been equilibrated with trichloromethane. The organic layer was dried under N_2, and the residue was dissolved in trichloromethane (200 μl). Aliquots of this solution served for determination of radioactivity.

Determination of labelled inositol phosphates

The inositol phosphates in the aqueous phase of the lipid extract were separated on AG 1×2 columns (2 ml) packed in Pasteur pipettes [23, 24]. Inositolphosphates were eluted from the column by a step gradient made up of 0.2, 0.4, 0.8 and 1 mol $\times 1^{-1}$ ammonium formate in 0.1 molar formic acid. Radioactivity was determined from aliquots of the eluate fractions (1 ml).

Elicitors

Glucan elicitors from *Alternaria carthami* [25] and from *Phytophthora megasperma* f. sp. *glycinea* [26] were prepared as previously described.

Elicitor-stimulation of parsley cells

To 6-day-old cell suspension cultures was added 0.5 μCi/ml of either [2-^{3}H]inositol, [2-^{3}H]glycerol or [^{32}P]orthophosphate. Twenty four hours later, aliquots (25 ml) of the suspensions were transferred into small flasks (100 ml), and Ac elicitor (0.1 mg/ml culture) dissolved in 1 ml water was added. Control cultures received 1 ml of water. When necessary, the cells were harvested by suction filtration and transferred immediately into test tubes containing the ice-cold trichloromethane-methanol-tetrabutylammonium hydrogen sulfate mixture. Lipids were extracted and separated as described above. Phosphoinositides labelled with either [2-^{3}H]inositol or [2-^{3}H]glycerol were spotted by radioscanning or visualized with molybdenum spray. Phosphoinositides labelled with [^{32}P]orthophosphate were separated by two-dimensional chromatography followed by autoradiography. Individual phosphoinositide fractions were scraped off the plate, and their radioactivity was determined by liquid scintillation counting in toluene containing 6 g diphenyloxazole and 330 ml Triton X-100/liter.

Elicitor-stimulation of soybean cells

60 to 80 g of soybean suspension cells (appr. 5 d after transfer) were removed from a medium having a conductivity of approximately 4 μS and placed in fresh B-5 medium modified in the following manner: the original myo-inositol content was reduced to 1/10th when [2-^{3}H]inositol labelling was performed and phosphate was completely omitted from the media for [^{32}P]orthophosphate labelling. After transfer to fresh medium, cultures were incubated with either 1.5 μCi/ml [2-^{3}H]inositol or 0.5 μCi/ml [^{32}P]orthophosphate for 10 h. After this time, aliquots (20 ml) of the suspension were pipetted into small flasks and *Pmg* elicitor (0.2 mg/ml suspension) was added. Control cultures received 1 ml water. Cells were harvested at appropriate times, and the phospholipids were extracted and separated, as described above.

Results

Detection of inositol lipids in parsley and soybean cell cultures

After labelling of either parsley or soybean cells for 24 h with [2-^{3}H]myo-inositol, the lipids were extracted from the cells according to a modified Bligh and Dyer-procedure [22]. All non-polar lipids migrated with the solvent front in subsequent one-dimensional chromatography of the lipid extract on oxalate-impregnated silica gel plates in solvent 1, whereas the inositol lipids were cleanly separated. Spots visualized by exposure of the plates to either iodine vapor [19] or molybdenum spray [20] revealed fractions which cochromatographed with authentic samples of PtdIns, PtdIns-4P, and PtdIns-4,5P_2 as reference. Radioscans of the lanes containing the

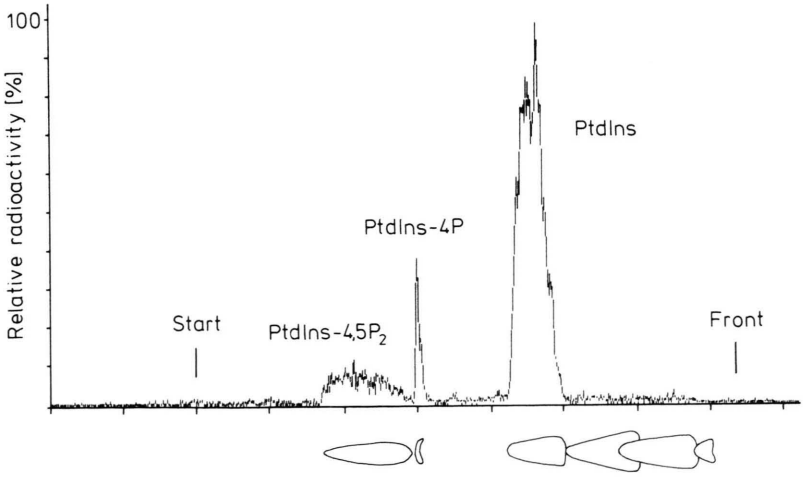

Fig. 1. Separation of [2-^{3}H]inositol-labelled phosphoinositides from cultured parsley cells on oxalate-impregnated silica gel plates with solvent system 1. Upper part shows scan of radioactivity. Lower part shows compounds spotted by a molybdenum spray.

[^{3}H]inositol-lipid extracts showed a distribution pattern corresponding to that of the reference inositol lipids (Fig. 1). The distribution of radioactivity in the phosphoinositides in parsley cells was approx. PtdIns 84%, PtdIns-4P 6%, and PtdIns-4,5P_2 8%. The R_f-values found for the phosphoinositides and for other phospholipids were in accordance with the values reported in the literature [27].

Phospholipids labelled for 24 h with [^{32}P]orthophosphate were also extracted from both parsley and soybean suspension cultures and were separated by two-dimensional chromatography on oxalate-impregnated silica gel plates and detected by subsequent autoradiography (Figs. 2A and 2B). In both cases the [^{32}P]phosphoinositides comigrated with the respective reference compounds, which were visualized by iodine vapor or with a molybdenum spray.

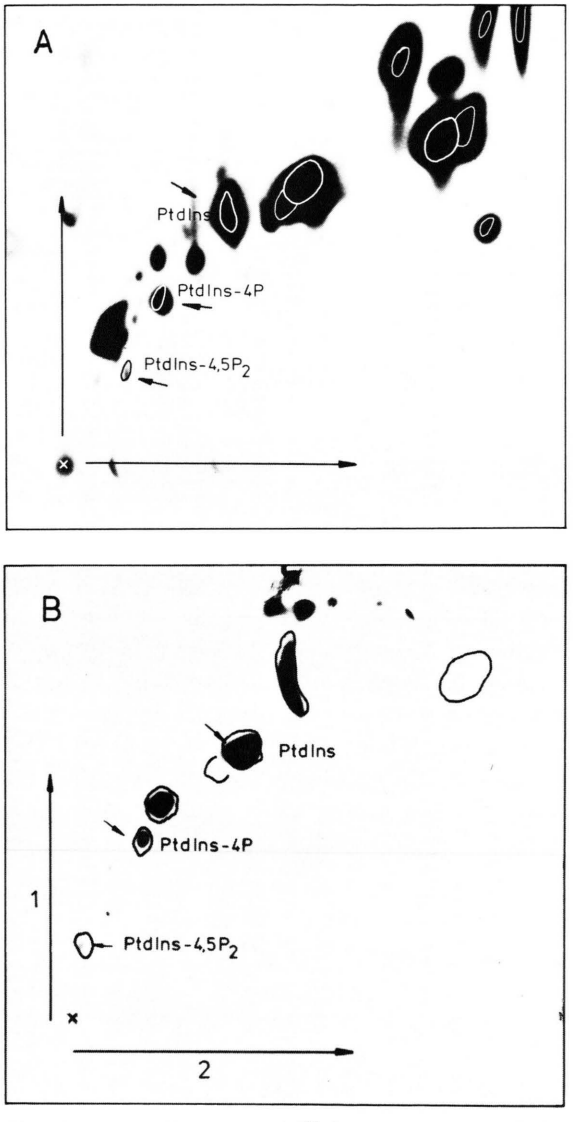

Fig. 2. Autoradiograms of [^{32}P]orthophosphate-labelled phospholipids that were isolated from (A) parsley cells and (B) soybean cells. The phospholipids had been separated by two-dimensional chromatography on oxalate-impregnated silica gel plates in trichloromethane-methanol-4N-ammonia 9:7:2 (dimension 1) and trichloromethane-acetone-methanol-acetic acid-water 45:15:13:10:7 (dimension 2), and phosphoinositides were identified by co-chromatography with authentic reference materials. The circles indicate the position of phospholipids visualized with molybdenum spray.

Deacylation of phospholipids and assay of labelled inositolphosphate fractions

For further characterization, the [^{3}H]inositolphosphoinositides isolated from soybean cells – were deacylated to the corresponding [^{3}H]glycerophosphoinositols, and the latter were purified by anion exchange chromatography [23]. Three radioactive fractions were separated by stepwise elution (Fig. 3), which represent glycerophosphoinositol, glycero-

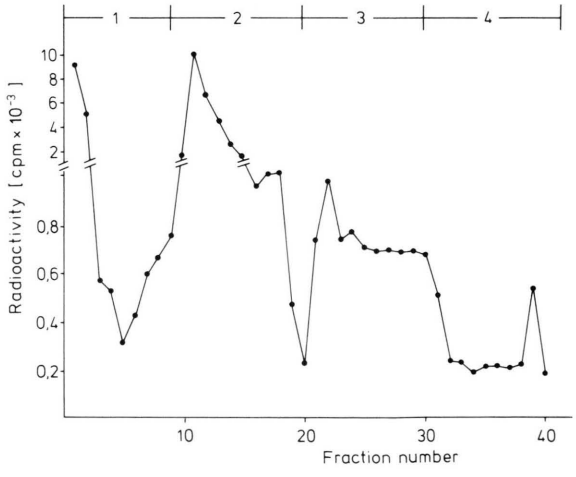

Fig. 3. Separation of deacylation products of [2-^{3}H]inositol-labelled phospholipids from soybean cells by ion exchange chromatography on AG 1×2 (formate form) resin. The glyceroinositols were eluted from the resin by a step gradient formed from (1) water, (2) 5 mM $Na_2B_4O_7$/0.18 M ammonium formate in water, (3) 0.3 M ammonium formate in 0.1 M formic acid, and (4) 0.75 M ammonium formate in 0.1 M formic acid. Under these conditions, glycerophosphoinositol is eluted first (gradient step 2), followed by its monophosphate (gradient step 3) and its bisphosphate (gradient step 4) [23].

phosphoinositol monophosphate and glycerophosphoinositol bisphosphate, respectively [23].

Labelled metabolites recovered from the aqueous phase after extraction of phospholipid from soybean cells that had been incubated with [^{32}P]orthophosphate for 24 h were also separated by anion exchange chromatography [24]. Most of the radioactivity was found in the fraction that was eluted with 0.4 M ammonium formate (Ins-P) (Fig. 4). Yet a significant amount of radioactivity was also found in the fractions corresponding to Ins-P_2 (gradient step 3), and a small amount of radioactivity was eluted with 1 M ammonium formate, indicating the presence of Ins-P_3 (Fig. 4) [24]. In similar experiments employing parsley cells, no exact correlation was found in chromatographic mobility of the isolated inositolphosphates with the R_f-values reported for such compounds in the literature [28, 29].

Effect of elicitor challenge on inositol lipid turnover

A. Parsley cell cultures

Parsley cells (7 day-old) which had been labelled for 24 h with either [2-^{3}H]inositol, [2-^{3}H]glycerol, or [^{32}P]orthophosphate were stimulated with Ac elicitor. In all of these experiments, labelling of phosphoinositides was examined after one-dimensional and also after two-dimensional chromatographic separation. As shown for example in Figs. 5A and B for [2-^{3}H]inositol incorporation, no significant differences in the relative labelling of PtdIns-4P and PtdIns-4,5P_2 was observed in elicitor-treated versus control cells. Labelling with [2-^{3}H]glycerol yielded very similar results (data not shown). Experiments employing [^{32}P]orthophosphate (Fig. 6) again revealed no significant differences in the incorporation of label into PtdIns-4P between elicitor-treated and control cells. In the latter experiments, the low incorporation of label into PtdIns-4,5P_2 did not allow a quantitative evaluation.

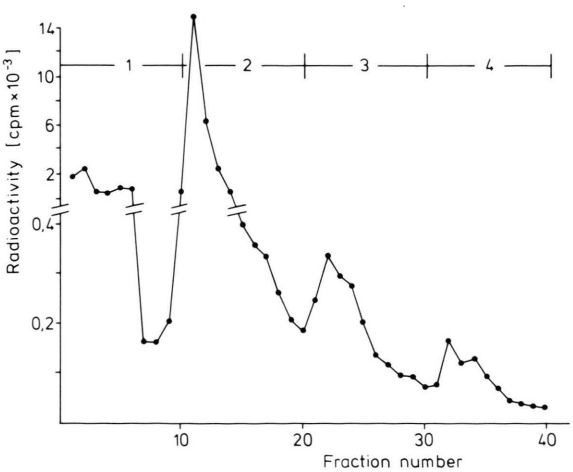

Fig. 4. Separation of inositol phosphate metabolites from the aqueous phase of the initial phospholipid extraction of [^{32}P]orthophosphate-labelled soybean cells. Separation was achieved by ion exchange chromatography on AG 1×2 resin in 0.1 M formic acid containing either (1) 0.2 M, (2) 0.4 M, (3) 0.8 M or (4) 1 M ammonium formate.

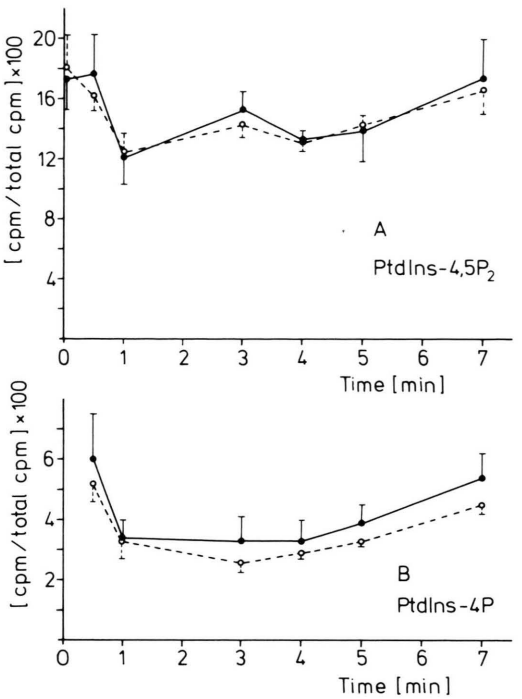

Fig. 5. Radioactivity incorporated into (A) PtdIns-4,5P_2 and (B) PtdIns-4P in parsley cells incubated with [2-^{3}H]inositol. 24 h after addition of the labelled inositol, either (○) water (1 ml/25 ml culture; control) or (●) Ac elicitor (2.5 mg dissolved in 1 ml water/25 ml culture) was added to the cell cultures, and the incubation was continued for various times as indicated. Amounts of radioactivity recovered in the polyphosphoinositides are indicated as % of total radioactivity in phosphatidylinositol. The polyphosphoinositides were separated as described in Fig. 1. Results are averages from three determinations ± SD.

B. Soybean cell cultures

In a first series of experiments 5 day-old soybean cells, which are in the second maximum for elicitor

inducibility [17], were used, whereas in later experiments cells at the stage of the first maximum for inducibility (10 h after transfer) were employed. Glyceollin accumulation by the cells was determined by HPLC analysis [30] for control of successful induction.

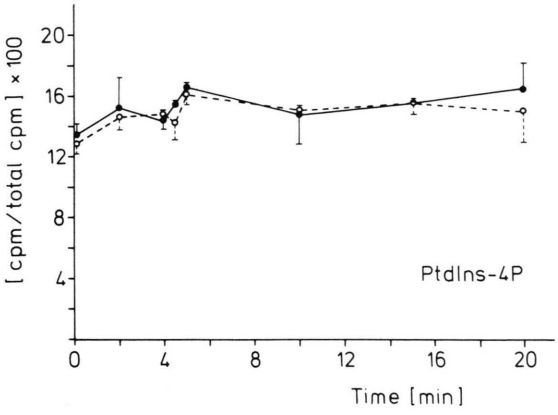

Fig. 6. Radioactivity incorporated from [^{32}P]orthophosphate into PtdIns-4P in parsley cells at various times after elicitor addition to the cell cultures. Conditions for controls (○) and elicitor induction (●) were as described under Fig. 5. The phospholipids were separated by two-dimensional chromatography, and amounts of radioactivity in the phosphoinositides are indicated as % of total radioactivity in phosphatidylinositol. Results are averages from three determinations ± SD.

Approximately 55% of the radioactivity was taken up by the soybean cells incubated for 10 h with [2-^{3}H]inositol. Whereas PtdIns-4P incorporated about 10% as much radioactivity as PtdIns, PtdIns-4,5P_2 became labelled to only a low extent under these conditions (Figs. 7A and B). Pmg elicitor was added to the cells after 10 h labelling. At 7 and 10 minutes after addition of the elicitor, control cells apparently incorporated more radioactivity into PtdIns and PtdIns-4P than elicitor treated cells (Figs. 7A and B). However, this result was not reproducible, and in some experiments slightly more radioactivity was recovered in the phosphoinositides from elicitor-treated cells as compared to the controls.

In different sets of experiments, the cells were incubated with [^{32}P]orthophosphate for 10 h prior to addition of *Pmg* elicitor. Again, over the period of 3 to 20 minutes after elicitor challenge, no significant differences in the amount of radioactivity incorporated into the inositol-lipids were observed between elicitor-treated cells and control cells (Figs. 8A and B).

Discussion

The occurrence of phosphatidylinositol 4-phosphate and of phosphatidylinositol 4,5-bisphosphate in cultured parsley and soybean cells was demonstrated by one- and two-dimensional co-chromatog-

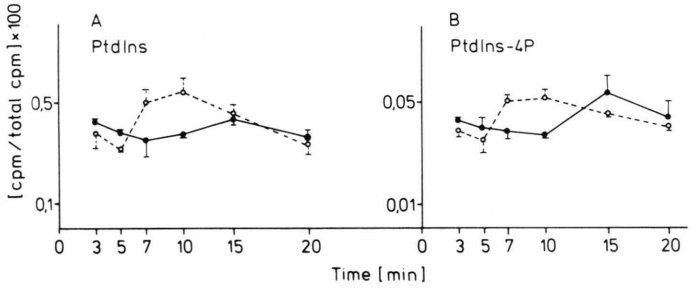

Fig. 7. Radioactivity incorporated from [2-^{3}H]-inositol into (A) PtdIns and (B) PtdIns-4P in soybean cells at various times after elicitor addition to the cell cultures. Conditions for controls (○) and elicitor induction (●) were as in Fig. 5. Amounts of radioactivity recovered in the phosphoinositides are indicated as % of total radioactivity extracted from the cells. Results are averages from three determinations ± SD.

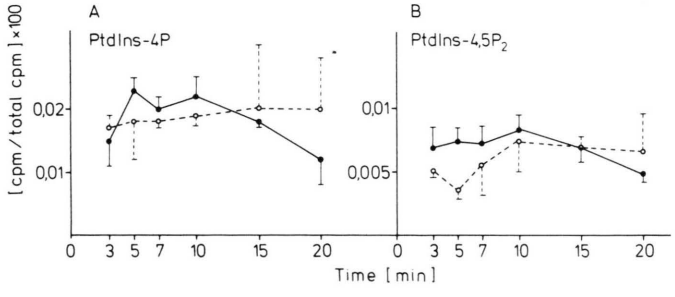

Fig. 8. Radioactivity incorporated from [^{32}P]-orthophosphate into (A) PtdIns-4P and (B) PtdIns-4,5P_2 in soybean cells at various times after elicitor addition to the cell cultures. Conditions for controls (○) and elicitor induction (●) were as in Fig. 6. Amounts of radioactivity recovered in the polyphosphoinositides are indicated as % of total radioactivity extracted from the cells. Results are averages from three determinations ± SD.

raphy with authentic reference polyphosphoinositides (Figs. 1 and 2). Incorporation of [2-^{3}H]inositol, [2-^{3}H]glycerol and [^{32}P]orthophosphate into the plant cell inositides further confirmed this identification. Additional confirmation was obtained by anion-exchange chromatography of the water-soluble products that resulted from chemical deacylation of the isolated inositides [31] (Fig. 3), and by comparison of the [^{32}P]orthophosphate-labelled inositol phosphate metabolites recovered from the aqueous phase of the initial plant cell phosphoinositide extraction (Fig. 4). However, we cannot exclude the possibility that the isolated polyphosphoinositides are isomers of those occurring in animal systems. In the meantime, the presence of polyphosphoinositides has also been reported from suspension-cultured *Daucus carota* [32] and *Catharanthus roseus* [33] cells.

In sets of experiments that were repeated several times, elicitor-challenge did not significantly influence the rate of incorporation of either [2-^{3}H]inositol, [2-^{3}H]glycerol or [^{32}P]orthophosphate into the polyphosphoinositides of parsley (Figs. 5 and 6) and soybean (Figs. 7 and 8) cells. The extraction of the phospholipids was done as soon as 30 s following addition of the elicitor to the cells. Owing to difficulties in experimental handling, however, extraction was routinely started not earlier than 1–2 minutes after elicitor addition.

In pituitary and blood cells, for example, maximal polyphosphoinositide breakdown may occur within 15 s [34], and usually between 30 and 60 s [35], in response to a hormone stimulus. The fastest effects of elicitors on cultured plant cells reported so far include changes in the plasmalemma permeability [11] and the activation of genes encoding "pathogenesis related proteins" (PR genes) [36] in parsley. Phosphate uptake of these cells became impaired within 2–3 minutes, and a 4-fold increase in the transcriptional rate of the PR-1 gene was observed within 5 minutes following elicitation. On the other hand, an increased rate of transcription of the 4-coumarate:CoA ligase gene was observed at 1 h after addition of elicitor to the parsley cells, which reached its maximum at 1.5 h [7, 8]. Furthermore, elicitor-induced inhibition of phosphate uptake into parsley cells could be fully reversed if the elicitor was washed out within a few minutes following its addition to the cells [11]. When the elicitor was present for at least 20 minutes, however, its effect on the cells was no longer reversible [10].

The results summarized above suggest that any possible modulation of the plant cell polyphosphoinositide turnover in response to elicitor should become prominent within at least 20 to 30 minutes following addition of the elicitor to the cells. We conclude, therefore, that polyphosphoinositides are not involved in the signal transmission in elicitor-challenged parsley and soybean cells.

Acknowledgements

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