Evidence of an Auxin-Mediated Phosphoinositide Turnover and an Inositol (1,4,5)Trisphosphate Effect on Isolated Membranes of *Daucus carota* L.

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Microsomal membranes from carrot suspension cells were phosphorylated in vitro with $[\gamma\text{-}^{32}P]ATP$. In the presence of submicromolar concentrations of the natural auxin indoleacetic acid (IAA), a rapid, but transient decrease of the $[^{32}P]$ label could be detected in the phospholipid extracts of the membranes. The phytohormone effect was not the result of an inhibition of the lipid phosphorylation reactions, but was caused by a simultaneous release of water-soluble compounds, which, according to their chromatographic properties, were assumed to contain inositol polyphosphates. Although the $[^{32}P]$-labeled lipids, as well as the inositol polyphosphates, were not identified unequivocally by chemical analysis, these findings point to an auxin-mediated control of a phosphoinositidase C-like reaction similar to the hormone-stimulated phosphoinositide response in animals. Exogenously applied inositol (1,4,5)trisphosphate $[(1,4,5)IP_3]$ was found to release $^{45}Ca^{2+}$ from preloaded membrane vesicles of carrot cells. Both the detection of the auxin-stimulated phosphoinositide response and the $(1,4,5)IP_3$-mediated Ca^{2+} release on isolated cell membranes offer new experimental approaches for the identification of the putative auxin receptor and its signal transduction pathway.

Key words: auxin action, phosphoinositide phosphorylation in vitro, Ca^{2+} release

Several authors have interpreted their findings on the thin-layer chromatography (TLC) analysis of lipid extracts to mean that higher plant cells contain phosphatidylinositol (PI) 4-monophosphate and phosphatidylinositol 4,5-bisphosphate, i.e., (4)PIP and (4,5)PIP_2 [1–4]. Like animal cells, the polyphosphoinositides are localized mainly at the plasma membrane [4] and are formed by specific lipid kinases phosphorylating PI and (4)PIP [5]. Also, there is increasing evidence for the occurrence in plant cells of the phosphoinositidase C [6,7], the protein kinase C [8], and the Ca^{2+}-mobilizing activity of inositol (1,4,5)trisphosphate $[(1,4,5)IP_3]$ [9–13]. All these compounds and their enzymes are involved as functional elements in the signal-dependent

phosphoinositide response (PI response) in animals [14]. Consequently the PI response is now also considered a signal-transducing mechanism in plants [15,16]. However, to date there has been less evidence coupling these elements into such a plant signal processing system. Putative signals for the initiation of the PI response in plants include light, with its stimulating effects on gravitropism [17,18] and on leaflet movement [19], and phytohormones [20–23]. Early reports describe a release of bound calcium [24,25] and phosphate [26] from isolated plant membranes by the action of the auxin indoleacetic acid, a natural phytohormone. The phenomenon was interpreted to be a consequence of a hormone-mediated degradation of anionic phospholipids. In this connection the finding of an auxin-mediated loss of PI was explained by the hormone's action on the inositol exchange reaction [27], but recent findings point instead to the action of auxin on the PI turnover via a phosphoinositidase C-like reaction [20,21,23]. A further confirmation of this view is presented here by results demonstrating an auxin-mediated formation of inositol phosphate-containing compounds and the putative capacity of (1,4,5)IP$_3$ to release sequestered ^{45}Ca^{2+} from microsomal membranes of carrot suspension cells.

MATERIALS AND METHODS
Chemicals

[γ-^{32}P]ATP (0.55 or 1.07 TBq mmol^{-1}) and [^{45}Ca]Cl$_2$($\approx$1 GBq mg^{-1} Ca) were purchased from the Radiochemical Centre Amersham (Buckinghamshire, Great Britain). (1,4,5)IP$_3$ was obtained from Boehringer (Mannheim, FRG), Calbiochem (Frankfurt, FRG; lot no. 795029), and Sigma (Deisenhofen, FRG; lot no. 65-F84432), whereas (1,4)IP$_2$ was from Sigma (Deisenhofen, FRG; lot no. 65F-34232). All other chemicals were of analytical grade and were obtained from several sources.

Membrane Preparation

Microsomal membranes were prepared as described [20,23] from carrot cells grown in suspension cultures. Immediately after homogenization of the cells, the extract was centrifuged at 50,000g for 60 min, and after resuspension the membranes were fractionated in discontinuous Renografin density gradients by centrifugation in a swinging-bucket rotor for 2 h. The membrane fractions were washed three times with buffer, and a concentrated membrane suspension was stored in liquid nitrogen until use.

PI Phosphorylation Assay

The standard assay contained 93 kBq [γ-^{32}P]ATP, 100 μM Na$_2$ · ATP, 10 μM GTP, 10 mM MgSO$_4$, 25 mM LiCl, 125 μg membrane proteins in a final volume of 500 μl buffer at pH 7.5. The reaction was started by the addition of an aliquot of the ATP/GTP mixture, incubated at room temperature, and terminated by the addition of 1 ml ice-cold stop solution containing 2-propanol/conc. HCl (100/1 v/v).

Lipid Extraction and Fractionation of Inositol Phosphates

The lipids were extracted from the acidified propanolic solutions with the solvent system using n-hexane as organic solvent according to Hara and Radin [28]. Lipids were separated by TLC on silicagel 60 plates using an acidic solvent system composed of chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, v/v). After lipid

extraction the inositol phosphates of the aqueous phases were separated by anion exchange on Dowex AG 1-X2 (200–400 mesh) resin either by the use of a batch mode or by chromatography on 1 ml columns as described [29]. [^{32}P] label of the extracts was measured via Cerenkov radiation in a liquid scintillation counter or by autoradiography of TLC plates exposing Kodak X-Omat x-ray film for 10 days.

$^{45}Ca^{2+}$ Efflux From Preloaded Membrane Vesicles

For Ca^{2+} accumulation, microsomal membrane vesicles (100 μg protein) were incubated for 30 min at +25°C in a final volume of 250 μl medium with 50 μM $^{45}CaCl_2$ (4.33 kBq), 1 mM Na_2 · ATP, 3 mM $MgSO_4$, 50 mM KCl, 100 μM NaN_3, 25 mM Pipes/KOH (pH 7.0), and 250 mM sucrose. The reaction was stopped by a rapid vacuum filtration of a 200 μl aliquot through nitrocellulose filters (0.45 μm). The filter was washed with 3 ml buffer (25 mM Pipes/KOH, pH 7.0, 250 mM sucrose, 10 mM $CaCl_2$), and its radioactivity was determined after the addition of a scintillant mixture by liquid scintillation counting. For $^{45}Ca^{2+}$ efflux studies, the Ca^{2+} accumulation by vesicles was stopped by the addition of 2 mM EGTA, and 30 s later (1,4,5)IP$_3$ or H_2O were added for a further 30 s incubation, before the assay was terminated as described. Experiments were performed as triplicate assays, and the results presented refer to the ATP-dependent Ca^{2+} accumulation.

RESULTS

Membranes prepared from carrot suspension cells exhibit the capacity for a rapid lipid phosphorylation in vitro. The endogenous activity was found to be comparable with the findings on membranes of animal cells. The lipid phosphorylation can be saturated with increasing ATP concentration and depends absolutely on the presence of Mg^{2+}, indicating Mg · ATP as the true enzymatic substrate [20,23]. In order to analyze the products of the in vitro incubation of microsomal membranes with [γ-^{32}P]ATP, the lipid extract was separated by TLC, and four spots of [^{32}P]-labeled phospholipids were detected by autoradiography (Fig. 1). These compounds were identified as phosphatic acid (PA), lysophosphoinositide monophosphate (LPIP), (4)PIP, and (4,5)PIP$_2$ by comparison of their chromatographic behavior with published R$_f$ values for the acidic solvent system being used. Only weak [^{32}P] labels were detected in the phosphoinositides, whereas the strong radioactive incorporation in PA was obviously caused by a high endogenous diacylglycerol kinase (EC 2.7.1.-) activity. The substrate for the latter reaction might be generated during membrane preparation by the combined action of phospholipase D (EC 3.1.4.4) and PA phosphatase (EC 3.1.3.4) according to Scherer and Morré [30].

The lipid phosphorylation detected in vitro has provided the experimental approach to an analysis of a putative auxin effect on the PI turnover in isolated carrot cell membranes. First the kinetics of the lipid phosphorylation in the absence or presence of 1 μM IAA were analyzed. In comparison with control conditions, a very fast, but transient reduction of [^{32}P] label could be observed in the lipid fraction of the hormone-treated assays at 30 s, but not at longer incubation periods (Fig. 2). The fall in [^{32}P] label is not caused by an auxin-mediated inhibition of the PI phosphorylation reaction, but is generated by an auxin-mediated hydrolysis of [^{32}P]-labeled PIs, since [^{32}P]-labeled compounds could be detected in the aqueous extracts after their fractionation by anion exchange chromatography (Fig. 3). According to their elution positions
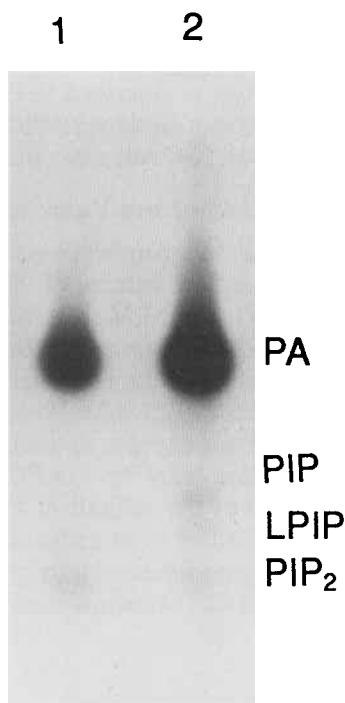
Fig. 1. Autoradiograph of membrane lipids in vitro phosphorylated with [γ-^{32}P]ATP. Microsomes were incubated on standard conditions for 30 s and 120 s. The lipids were extracted and separated by TLC.

these compounds are assumed to contain IP_2 and IP_3, but not ATP. In general, it is difficult to demonstrate any release from membranes of $(1,4,5)IP_3$ as a product of $(4,5)PIP_2$ hydrolysis by the phosphoinositidase C, if the phosphoinositides were labeled with [γ-^{32}P]ATP. In this case it is known that the [^{32}P]-labeled IP_3 coelutes with the nucleotide in the commonly used chromatography systems. However, as demonstrated in a previous study, a contamination of [^{32}P]-labeled IP_3 by [^{32}P]ATP in the
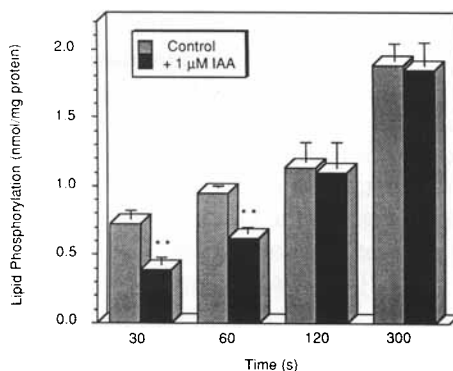

Fig. 2.   Kinetics of the lipid phosphorylation of microsomal membranes in the absence and presence of 1 μM IAA. The results shown are mean ± SD (**$P < 0.01$, Student's unpaired t test).
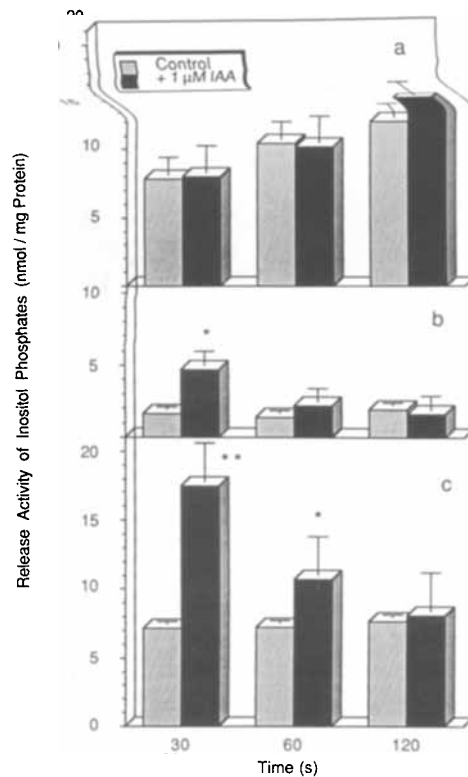
Fig. 3. Kinetics of [^{32}P]-labeled compounds derived from microsomal membranes that were incubated in the absence or presence of 1 μM IAA. [^{32}P]-labeled fractions containing PI and IP (a), IP$_2$ (b), and IP$_3$ (c) were separated by anion exchange chromatography on small columns. The results shown are mean ± SEM (*$P < 0.10$, **$P < 0.05$, Student's unpaired t test).

elution profile of the anion exchange chromatography used could be nearly excluded. The bulk of [^{32}P]ATP was found to be eluted at a higher ionic strength than [^{32}P]-labeled IP$_3$ [23].

Another indication is based on the kinetics of the auxin-mediated release of the [^{32}P]-labeled compounds, since the time course corresponds well with the kinetics of the loss of the [^{32}P] label in the lipid fraction (Fig. 3). Surprisingly, the absolute [^{32}P] label of IPs being released exceeded that of the hormone-mediated loss of lipid phosphorylation. This discrepancy can be explained as a consequence of the method used. The [^{32}P] label of the lipids reflects the current state of the lipid pool being phosphorylated, but the radioactivity of the IPs results from the accumulation of the compounds being released during the whole incubation period. The auxin-stimulated PI response also exhibits a dose-response relationship. Corresponding to a continuous loss of [^{32}P] label of the phospholipids [20,23] a hormone-stimulated release of [^{32}P]-labeled IPs was observed with increasing IAA concentrations (Fig. 4). In the experiment shown, increasing IAA concentrations caused a nearly linear increase of released IP$_2$, but a progressive release of IP$_3$ up to a saturation level at 1 μM hormone. This phenomenon may reflect differences in the turnover of the distinct IPs. Finally, the presence of
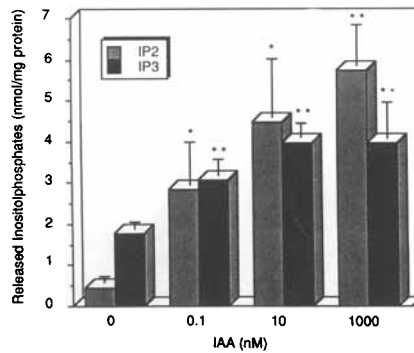
Fig. 4.   Dose-response effect of IAA on the release of water-soluble [^{32}P]-labeled compounds during a 30 s incubation of microsomal membranes. The fractions containing IP$_2$ and IP$_3$ were separated by anion exchange chromatography using the batch mode. The results shown are mean ± SEM (*$P < 0.05$, **$P < 0.01$, Student's unpaired t test).

guanine nucleotides was found to promote slightly the auxin-mediated reaction in some but not all assays (data not shown).

In animal cells (1,4,5)IP$_3$, as the cytosolic product of the signal-dependent hydrolysis of (4,5)PIP$_2$ by the phosphoinositidase C, acts as a second messenger for the release of Ca^{2+} from internal stores [14]. It was reported that microsomal vesicles from carrot suspension cells are able to accumulate ^{45}Ca^{2+} in an ATP-dependent manner [31], and (1,4,5)IP$_3$ was found to mobilize ^{45}Ca^{2+} from fusogenic carrot protoplasts [11]. Consequently, we were interested in looking for a similar potency of (1,4,5)IP$_3$ on microsomal membranes prepared by us from carrot suspension cells. Indeed, after the addition of commercially available (1,4,5)IP$_3$ ^{45}Ca^{2+} was released
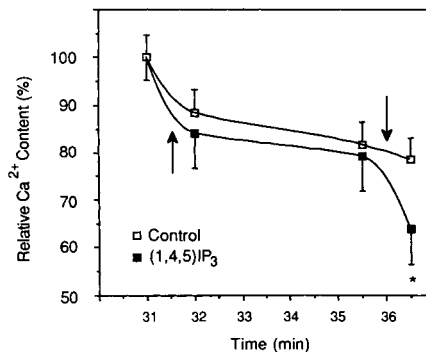

Fig. 5.   Kinetics of the (1,4,5)IP$_3$-mediated Ca^{2+} release from preloaded microsomal membrane vesicles. For these experiments the assay volume was quadrupled. After a 30 min incubation for Ca^{2+} loading, EGTA (2 mM final concentration) was added to the assays, and at the time points indicated by the arrows H$_2$O or (1,4,5)IP$_3$ (20 μM final concentration) were applied. Both 30 s before and after these additions a 200 μl aliquot was withdrawn and vacuum-filtrated. The value of 100% Ca^{2+} accumulation corresponds to 3.5 nmol Ca^{2+}/mg protein. The results shown are mean ± SD (*$P \approx 0.06$, Student's unpaired t test).
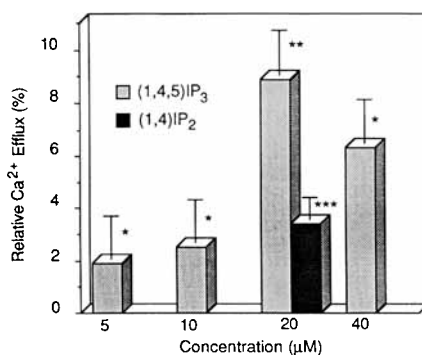
Fig. 6. Dose-response effect of $(1,4,5)IP_3$ on the Ca^{2+} release from preloaded microsomal membrane vesicles. After Ca^{2+} accumulation for 30 min, EGTA (final concentration 2 mM) was added to 500 μl assays. For the determination of accumulated Ca^{2+} a 200 μl aliquot was withdrawn 2.5 min later, before inositol polyphosphates or H_2O as control were applied to the assays. After 30 s a second 200 μl aliquot was taken out and vacuum-filtrated. Results are expressed as the relative extent of the inositol polyphosphate-mediated Ca^{2+} efflux in comparison with the ATP-dependent Ca^{2+} accumulation of the H_2O control. The results shown are mean $\pm$ SD (*$P < 0.05$, **$P < 0.02$, ***$P < 0.01$, Student's unpaired t test).

within few seconds from membrane vesicles, which were preloaded using ATP as energy substrate (Fig. 5). The extent of the $(1,4,5)IP_3$-mediated response varied between different assays, and the variation might be caused by endogenous phosphatase activity leading to the degradation of inositol phosphates. In accordance with this variability, it was difficult to determine any significant difference in the potency of $(1,4,5)IP_3$ obtained from the three different commercial sources. However, increasing the transmembrane Ca^{2+} gradient either by reducing the concentration of free Ca^{2+} outside the preloaded vesicles by a higher EGTA concentration or an extension of the preincubation period with the chelator was found to double the extent of the $(1,4,5)IP_3$ response (data not shown). Also, a second pulse of $(1,4,5)IP_3$ was obviously more potent for releasing accumulated $^{45}Ca^{2+}$ (Fig. 5), which might be an indication for a temporally limited increase of the Ca^{2+} conductance of the vesicle membrane. A dose-response relationship as well as some specificity of the $(1,4,5)IP_3$-mediated $^{45}Ca^{2+}$ efflux could be detected, whereas relatively high concentrations of 20 μM $(1,4,5)IP_3$ were needed to initiate a significant response (Fig. 6). Despite the obvious potency of IAA for releasing inositol polyphosphates from carrot microsomal membranes, we could not detect any significant phytohormone-mediated release of $^{45}Ca^{2+}$ from preloaded membrane vesicles. This finding can be explained by the low amount of IP_3 that is generated by the auxin action and—as calculated from the data—is far below the concentration needed to stimulate Ca^{2+} release from membrane vesicles.

DISCUSSION

Isolated plant membranes can be used as an in vitro system for the analysis of lipid phosphorylations leading to the formation of polyphosphoinositides [5,20,23,32, this report] and for studies on their breakdown by a phosphoinositidase C-like reaction

with the subsequent release of inositol polyphosphates [6,20,23, this report]. Although recent reports point to the occurrence of (4)PIP and (4,5)PIP$_2$ [1–4,21] as well as (1,4,5)IP$_3$ [19,21] in plant extracts, the identification of distinct polyphosphoinositides and inositol polyphosphates have been performed by chromatographic methods. This standard approach was also used for the analysis of the in vitro phosphorylated lipids as well as for the water-soluble compounds released from the carrot microsomal membranes by the auxin-mediated effect. Recently, the occurrence of (4,5)PIP$_2$ in plant cells has been questioned by an isotopic analysis of phospholipids from tomato suspension cells [33] as well as by a chromatographic study demonstrating a possible comigration of (4,5)PIP$_2$ and LPIP in some solvent systems [4]. Chemical methods, i.e., deacylation of the polyphosphoinositides and subsequent conversion of the polar head groups to specific polyols by periodate oxidation, reduction, and dephosphorylation, must be applied for the definite identification of the substrates and products of the phosphoinositidase C-like reaction, as was recently done for the clear identification of (3)PIP, a new phosphoinositide detected in transformed animal cells [34].

Other open questions concern the precise subcellular localization of the microsomal membranes carrying the auxin-stimulated PI response as well as the (1,4,5)IP$_3$-mediated Ca^{2+} efflux. The kinases for the phosphorylation of PI and (4)PIP [5] and the phosphoinositidase C [6] are localized in the plasma membrane in plant cells. In this connection our results showing an auxin-mediated PI response point to an occurrence of the putative auxin receptor at the plasma membrane, and this view is accordance with the detection of auxin binding sites in plasma membranes by immunofluorescence [35] and photoaffinity labeling [36–38]. Recent investigations on the (1,4,5)IP$_3$-mediated Ca^{2+} efflux favor the vacuole, but not the endoplasmic reticulum as the intracellular compartment for this response in plant cells [12,13]. Another question about auxin transduction mechanism on membranes concerns the putative role of a G-protein known to be involved in the signal-dependent PI response in animal cells [39]. Our findings of a slight promotional effect of GTP for the auxin-mediated PI response coincide with reports that G-nucleotides were found to release inositol polyphosphates from plant membranes [40,41], and that they are bound at high-affinity sites on microsomal membranes [42–45].

In conclusion, to our knowledge this report is the first demonstration for a plant species that suggests the occurrence of both a hormone-mediated PI response on microsomal membranes and a Ca^{2+} efflux by (1,4,5)IP$_3$ from preloaded membrane vesicles. These findings are certainly important with respect to an elucidation of whether the phosphoinositide turnover is involved in signal transduction processes on plant membranes, as is known to occur in animal cells [14]. According to previous reports [20,21,23] our data can be considered as an additional indication, but not clear proof of the existence of an auxin-promoted PI response in higher plant cells. Beside the uncertainty of the unequivocal chemical identification of the inositol phospholipids and polyphosphates, there are other gaps that hinder our actual understanding of the signal transduction mechanism of a PI response on plant membranes. For future research the experimental system presented by us will be a useful tool for the in vitro analysis of auxin transmembrane signaling, which might coordinate action between an auxin receptor, a GTP-binding protein, and phosphoinositidase C [45]. Using in vivo experiments, it should be possible to answer the important physiological questions of how the auxin-mediated PI response is connected as a fast primary reaction to auxin-promoted long-term responses like plant cell division and elongation growth [46].

ACKNOWLEDGMENTS

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