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A RAPID RESPONSE TO A PLANT HORMONE: AUXIN STIMULATES PHOSPHOLIPASE A_2 IN VIVO AND IN VITRO

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SUMMARY: Addition of the active auxins indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid or α-naphthylacetic acid to cultured soybean (Glycine max L.) cells prelabeled with ethanolamine or choline increased the radioactivity in the lysophosphatidylethanolamine (LPE) or lysophosphatidylcholine (LPC) pool within 5 min. The inactive auxin analogue, β-naphthylacetic acid, was inactive in this response. In membranes prelabeled in vivo, either with ethanolamine or choline, and subsequently isolated from zucchini (Cucurbita pepo L.) hypocotyls, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid stimulated the conversion of phosphatidylethanolamine (PE) to LPE and of phosphatidylcholine (PC) to LPC in vitro whereas the inactive auxin analogue 2,3-dichlorophenoxyacetic acid did not.

We have shown that the lysolipid-like phospholipid, platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) stimulates a membrane-associated plant protein kinase and H^+-ATPases (1-5). More recently, also LPC and LPE were found to be active in these two assays (6). This prompted our search for a stimulus of plant phospholipase A_2 activity which would generate lysophospholipids and in turn would activate the protein kinase found by us (4,6). Our results show that biologically active auxins stimulate phospholipase A_2 in vivo and in vitro whereas inactive analogues were ineffective.

MATERIALS AND METHODS: Soybean cells (Glycine max L.) were grown in suspension culture (5) and used 5 d after inoculation in the late logarithmic phase of growth. A 100 ml culture was transferred into auxin-free medium for 18 h and then 2 µCi ^{14}C-ethanolamine or ^{14}C-choline (Amersham, Braunschweig, FRG) was added. After 3 h the medium was removed by gentle suction and the cells were washed with a small volume of fresh auxin-free medium. The cells were divided into two cultures of 40 ml

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each, one with $5x10^{-4}$ or 10^{-3} M hormone, one without. Samples of 5 ml were withdrawn, spun down rapidly and the cells suspended in 4 ml chloroform/methanol 1:2 (v/v) and left for 30-60 min at room temperature. Then 1 ml 0.1 M KCl was added, the resulting chloroform phase was dried *in vacuo* and dissolved in a small volume of chloroform for thin layer chromatography.

Zucchini (*Cucurbita pepo* L.) seedlings were grown for 4 d in the dark at 25-30 °C to 3-5 cm length. Pieces of 1 cm length were cut with a razor blade from the hypocotyl hook downward and 5 g tissue incubated in 10-20 µCi ^{14}C-ethanolamine or ^{14}C-choline in 6 ml water for 4 h at room temperature in the dark. The sections were rinsed with water and homogenized together with 20 g freshly harvested unlabeled sections (tissue/buffer ratio 1/1 (w/v)) in 0.5 M sucrose, 1 mM EDTA, 1 mM dithioerytritol and 15 mM Tris/HCl pH 7.5. The homogenate was centrifuged for 10 min at 10,000 g and the supernatant again for 30 min at 50,000 g. The resulting pellet was resuspended in 18 ml incubation buffer consisting of 250 mM sucrose, 250 mM 2-(N-morpholino)ethanesulfonic acid (Mes/Na^+) pH 5.5 and 1.5 mM $MgCl_2$. Auxins were added from 0.1 M ethanolic stock solutions at a concentration of 2×10^{-5} M and the same amount of ethanol was added to controls. Assays were incubated at 25 °C and samples of 1 ml were taken at appropriate time intervals, extracted by the procedure of Bligh and Dyer (7) and dried *in vacuo* for processing by thin layer chromatography. For ^{14}C-choline incubations the procedure was slightly modified. Membrane pellets were resuspended in 3 ml incubation buffer. Samples of 0.1 ml were taken, extracted (7) and the whole chloroform phase was submitted to thin layer chromatography.

Thin layer chromatography was done as described (8). For quantitative analysis, a solvent system of chloroform/methanol/acetone/acetic acid/water 100/20/40/20/10 (by volume) was chosen. After completion of the chromatogram, the silica gel was scraped off and the radioactivity counted in a scintillation counter.

RESULTS

In soybean cell cultures or zucchini hypocotyls labeled for 2-4 h with ^{14}C-ethanolamine the major radioactive lipid was phosphatidylethanolamine (PE) containing about 90% of the radioactivity (Fig. 1). Other radioactive lipids were identified by two-dimensional thin-layer chromatography (7), subsequent autoradiography, treatment with iodine vapor and sprays sensitive for amino groups and phosphorus (not shown). Phosphatidylcholine (PC), lysophosphatidylethanolamine (LPE) and a phospholipid tentatively identified as N-acyl-phosphatidylethanolamine were the minor radioactive lipids. The latter one is a component of many plant seeds (9,10). When auxin-depleted ethanolamine-prelabeled cells were treated with 5×10^{-4} M or

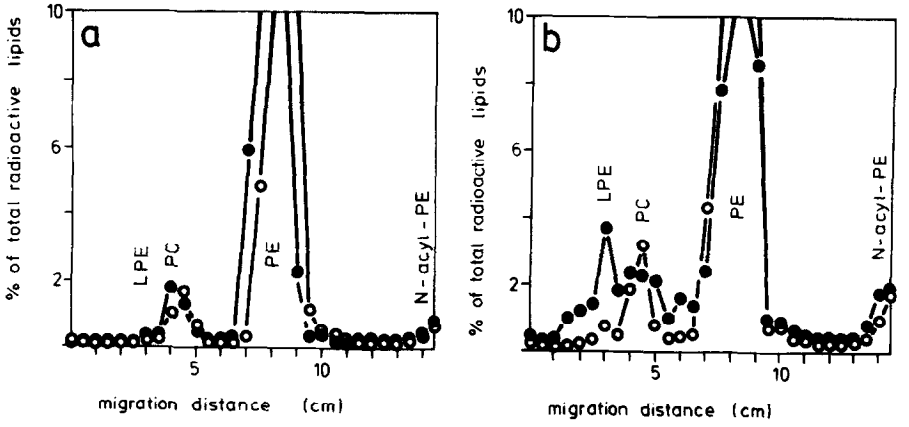

Fig. 1. Auxin-stimulated turnover of ^{14}C-ethanolamine-labeled phospholipids in cultured soybean cells. (a) Comparison of labeled products in the presence (●) and absence (○) of 10^{-3} M indole-3-acetic acid after 5 min chase of label. (b) Comparison of labeled products after in the presence (●) and absence (○) of 10^{-3} M indoleacetic acid after 90 min chase of label. Products were separated by thin-layer chromatography and 0.5 cm wide strips were counted.

10^{-3} M indole-3-acetic acid, a rapid redistribution of label with a decrease in PE and an increase in the other labeled phospholipids was observed (Fig. 1). The relative amount of radioactive LPE increased most dramatically to more than tenfold of the amount present at the start and more than fivefold

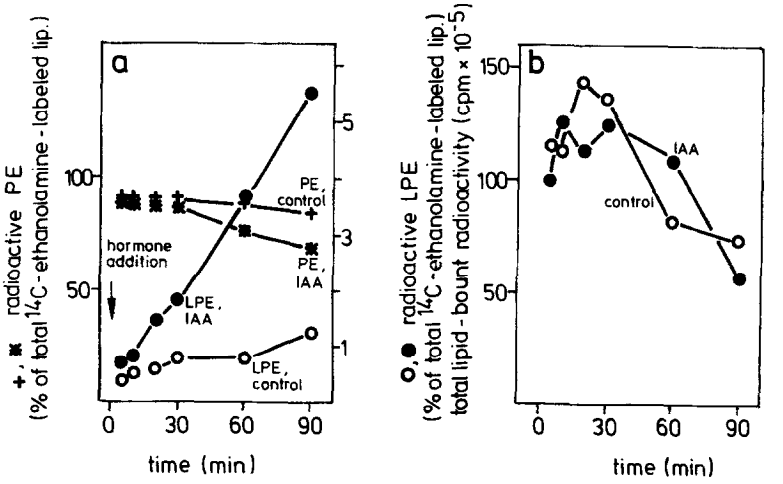

Fig. 2. (a) Kinetics of radioactive PE and LPE in the absence and presence of 10^{-3} M indole-3-acetic acid in cultured soybean cells prelabeled with ^{14}C-ethanolamine. (b) Kinetics of total lipid-bound radioactivity in cultured soybean cells prelabeled with ^{14}C-ethanolamine. Both panels show one and the same experiment.

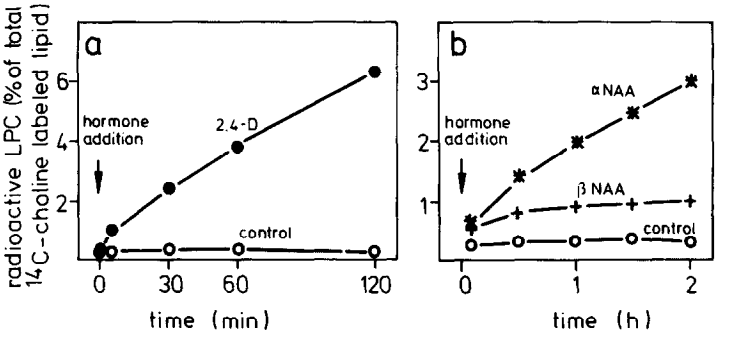

Fig. 3. Kinetics of radioactive LPC in the absence and presence of 5 x 10^{-4} M of the two active auxin analogues 2,4-dichlorophenoxyacetic acid (a) and α-naphthylacetic acid (b) and the inactive analogue β-naphthylacetic acid (b).

as in control cells without hormone (Fig. 2a) whereas the relative amount of radioactive PE decreased only little. The other two labeled products, PC and N-acyl-PE, increased only slightly during incubation with no clear hormone effect (not shown). The difference between the controls and hormone-treated cells in LPE content was apparent after 5 min and dramatic after 90 min whereas a different pattern in total lipid-bound radioactivity was not apparent (Fig. 2). When soybean cells were labeled with ^{14}C-choline, only PC and LPC were identified

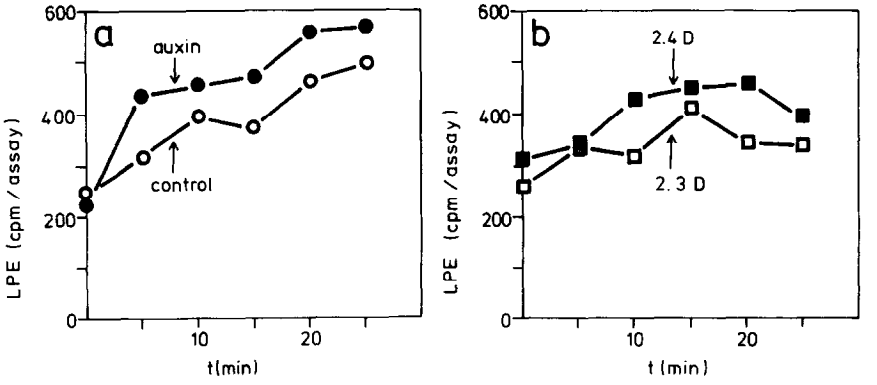

Fig. 4. Formation of lysophosphatidylethanolamine (LPE) in ethanolamine-prelabeled membranes isolated from zucchini hypoctyls. Labeled phospholipids were extracted after the incubation of the membranes with 2 x 10^{-5} M hormones and separated as shown in Fig. 1. The experiment shown in panel a and panel b were made simulataneously with the same batch of membranes.

as radioactive lipids (not shown). Biologically active auxins, 2,4-dichlorophenoxyacetic acid and α-naphthylacetic acid, applied to such cells again induced a rapid turnover of radioactivity from PC to LPC which was cleraly apparent after 5 min (Fig. 3) whereas the percentage of radioactive LPC in the presence of the inactive β-naphthylacetic acid and in the controls remained constant.

A stimulation of LPE production from prelabeled PE and of LPC production from prelabeled PC by auxins was also observed in isolated membranes (Fig. 4 and 5). Clearly, this indicates a stimulation of phospholipase A_2 by auxins. In vitro stimulation could be observed after 5 min and was specific for the native auxin indole-3-acetic acid and the biologically active analogue 2,4-dichlorophenoxyacetic acid at 2 x 10^{-5} M but not for the inactive analogue 2,3-dichlorophenoxyacetic acid. In experiments with five independent membrane preparations values for radioactive LPE increased after 5 min by 30% (± 16%) above the control level (=100% at t=0) and by 73% (± 9%) in assays with 2 x 10^{-5} M auxin.

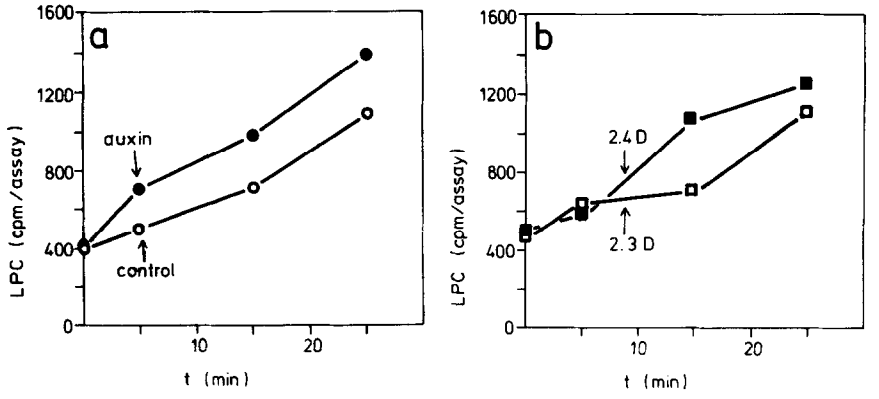

Fig. 5. Formation of lysophosphatidylcholine (LPC) in choline-prelabeled membranes isolated from zucchini hypocotyls. The hormone concentrations were 2 x 10^{-5} M and the thin layer chromatography of radioactive products was done as in Fig. 1.

DISCUSSION

For the first time, we demonstrate the stimulation of phospholipase A_2 by the plant hormone auxin in vivo and in vitro. This constitutes a new hormone-sensitive reaction which is apparent after 5 min and very rapid when compared to cell extension growth and proton extrusion, two other prominent responses of plant cells to auxin which typically exhibit a 10 min lag phase (11). Recently, the membrane-associated auxin receptor from maize has been shown to function in the signal transduction of the hormone response (12). Inasmuch as the stimulation of phospholipase A_2 is membrane-associated it could be part of the signal transduction mechanism as it is found in animal cells (13,14). This is further suggested by our finding that both LPC and, to a lesser extent, LPE are suitable to stimulate a membrane-associated protein kinase which phosphorylates substrate proteins in the tonoplast and the plasma membrane (6).

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