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Down-regulation by elicitors of phosphatidylcholine-hydrolyzing phospholipase C and up-regulation of phospholipase A in plant cells

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Abstract

Phosphatidylcholine, labeled by two fluorescent fatty acids, was fed to cultured plant cells (*Petrosilenum crispum*, L.; VBI-0, *Nicotiana benthamiana*, L.) and fluorescent diacylglycerol (DAG) was the major metabolite. When a glycoprotein elicitor, derived from *Phytophthora sojae*, was applied to the parsley cells and the small protein cryptogein from *Phytophthora cryptogea* was applied to the tobacco cells, these signal substances strongly and rapidly decreased the pool of fluorescent diacylglycerol and weakly increased the pool of free fluorescent fatty acid and of fluorescent lysophosphatidylcholine. The cells responded in a very similar way to the application of mastoparan, a wasp venom peptide. As phosphatidic acid was only a very minor fluorescent metabolite DAG is hypothesized to arise by the action of a phosphatidylcholine-hydrolyzing phospholipase C which was down-regulated by elicitors. Up-regulation of a phospholipase A by elicitors is also suggested by these results. This is the first evidence for phosphatidylcholine-hydrolyzing phospholipase C in plant signal transduction. © 2002 Elsevier Science (USA). All rights reserved.

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Plants and pathogens, when interacting, induce rapid signal transduction processes within and towards each other. In the plant this is a precondition for initiating defense reactions which usually culminate in the activation of a large number of genes. Substances used as a signal for the plant to recognize the presence of a pathogen are termed elicitors and may be derived from the host or the pathogen. A number of receptor proteins for elicitors have been identified and represent membrane and cytosolic proteins [1,2]. The signal transduction network activated by these elicitors is highly complex and may involve, besides many other proteins homologous to known signal components, phospholipase A (PLA), phospholipase C specific for phosphatidylinositol lipids (PI-PLC), and phospholipase D (PLD) [3,4]. The interactions of these components and their hierarchy, however, are not well understood at all nor is there a unified picture or scheme for these inter-

actions to be expected for the many pairs of hosts and pathogens.

We used the artificial phosphatidylcholine (PC), bis-BODIPY-PC, labeled by two fluorescent fatty acids, to feed it as a substrate to cultured plant cells to demonstrate the presence of an auxin- and elicitor-stimulated PLA since both stimuli lead to an accumulation of free fatty acids [5,6]. We noticed in both the controls and in auxin-treated cells a prominent fluorescent metabolite derived from the fluorescent PC which is identified here as diacylglycerol (DAG), suggesting a PC-hydrolyzing PLC (PC-PLC) to be also present in plant cells. Moreover, while auxin did not influence the level of this PC-derived metabolite, elicitor rapidly down-regulated it [6]. In animal cells phosphatidylcholine breakdown by a PC-PLC induced by growth factors and hormones has long been known but the eukaryotic gene for this enzyme has not yet been identified [7,8]. However, information on the regulation of a PC-PLC by plant-typical stimuli has been completely lacking. Here we report that two elicitors and the wasp venom peptide mastoparan rapidly down-regulated the level of DAG derived from PC so

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that the list of three established phospholipases in plant signal transduction, PLA [9], PI-PLC [10,11], and PLD [12] is extended for plants to include a PC-PLC.

Materials and methods

Cell cultures. The parsley (*Petroselinum crispum* L.) cell culture and partially purified raw elicitor [13] were obtained from Dr. D. Scheel (Institut für Pflanzenbiochemie, Halle, FRG) and grown in the dark in HA medium with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) [14]. At 26°C a growth cycle of 7 days was obtained. For the experiments cells were inoculated into auxin-free medium and used 3–4 days after inoculation in the logarithmic growth phase. Tobacco VBI-0 cells were obtained from Dr. E. Zázimalová (Institute for Experimental Botany, Prague) and grown as described [15]. Cryptogein was a gift from Dr. A. Pugin (University of Dijon, France).

Fluorescence labeling and metabolites. Cells grown to about 120–150 mg/mL fresh weight were sifted through a sterile kitchen tea sieve to discard cell clumps. To an appropriate volume of this culture a stock solution of 0.33 mg/mL bis-BODIPY-FL C₁₁-PC (1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine, Molecular Probes B-7701, MoBiTec, D-37077 Göttingen, Germany) in dimethyl sulfoxide was diluted 1:450 to 0.75 µg/mL. To samples of 1 mL proteinaceous agonists were added in water or auxin in ethanolic stock solution and the solvent was added up to equal amounts in all samples. All experiments were reproduced at least three times. Reactions were stopped by a modified Bligh and Dyer [16] extraction by 4 mL methanol/chloroform/conc. HCl = 150:75:1 (v/v/v) and 2 mL 0.1 M KCl was added subsequently. The organic phase was dried by a stream of nitrogen and redissolved in chloroform for thin-layer chromatography on Merck silica gel 60 in a solvent of tertiary butylmethylether/methanol/petrolether (b.p. 50–70°C)/water/conc. ammonia = 116/40/16/8.3/8.3 (by volume) (solvent

system 1; parsley extracts) or chloroform/methanol/water = 65/25/4 (by volume) (solvent system 2; tobacco extracts). For separation of neutral lipids the solvent system 3 was used consisting of petrolether (b.p. 50–70°C)/tertiary butylmethylester/methanol/acetic acid = 150/50/20/1 (by volume). Standards (FA, LPC, DAG) were prepared by digestion of fluorescent lipids by bee venom PLA₂ or bacterial PC-PLC (Sigma) and subsequent lipid extraction, while the fluorescent standards for sterol ester and PA were commercially available (MoBiTec, D-37077, Göttingen). Plates were dried and scanned optically by a video camera under UV light for computer-assisted quantification. In figures the fluorescence is shown as dark spots. To determine the relative stimulation increases in controls between $t = 0$ and 90 min were set as 100% and activities in the presence of agonists were calculated relative to this.

Results and discussion

Elicitor glycoprotein from *Phytophthora sojae* was added as a stimulus to parsley cells where it elicited a hypersensitive type of response [17]. Two fluorescent metabolites increased above the control levels with time, fatty acid (FA) to 210% at 60 min and lysophosphatidylcholine (LPC) to 120% at 30 min and 105% at 60 min. Diacylglyceride (DAG) decreased relative to the control to 25% at 30 min and at 60 min after elicitor treatment (Fig. 1A). The accumulation of these two metabolites in elicitor-treated samples is indicative of a rapid activation of PLA in the parsley cells whereas the decrease of DAG can be interpreted as a down-regulation of PC-PLC.

These fluorescent metabolites were identified by one-dimensional thin-layer chromatography in a solvent

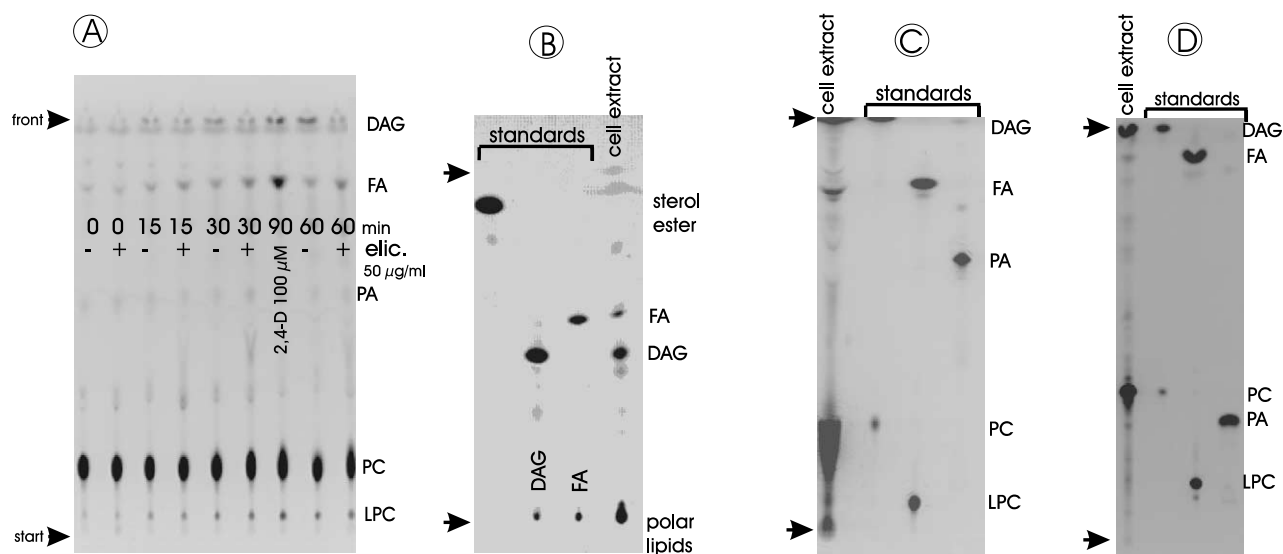


Fig. 1. (A) Thin-layer chromatography of lipids extracted from parsley cells treated with raw elicitor preparation from *P. sojae* in a time course experiment. Solvent system 1 was used and only fluorescent compounds are visible and identified compounds are indicated. DAG = diacylglyceride; FA = free fatty acid; PA = phosphatidic acid; PC = phosphatidylcholine; LPC = lysophosphatidylcholine. (B) Thin-layer chromatography of lipids extracted from parsley cells incubated for 90 min with bis-BODIPY-PC. Solvent system 3 was used for separation of neutral lipids and fluorescent compounds were identified by comparison to standards as indicated. (C) Thin-layer chromatography of lipids extracted from parsley cells incubated for 90 min with bis-BODIPY-PC. Solvent system 1 was used and fluorescent compounds were identified by comparison to standard compounds as indicated. (D) Thin-layer chromatography of lipids extracted from tobacco VBI-0 cells incubated for 90 min with bis-BODIPY-PC. Solvent system 2 was used and fluorescent compounds were identified by comparison to standard compounds as indicated.

system suitable to separate neutral lipids (Fig. 1B) and polar systems as shown here (Fig. 1C, D) by two-dimensional thin-layer chromatography [5] and HPLC (data not shown). Sterol ester was excluded as a potential fluorescent metabolite. Additionally, the position of fluorescent phosphatidic acid (PA), another potential metabolite, was identified in the polar solvent systems used here (Fig. 1C, D). This showed, by comparison, that fluorescent PA was an insubstantial fluorescent metabolite if present at all (Figs. 1 and 2). In contrast to elicitor treatment, fluorescent DAG was not changed by the auxin 2,4-D. Auxins activated a phospholipase A activity and increased the FA pool strongly (Fig. 1A) but the increased LPC pool consistently only at very high concentrations above 100 μM auxin [5].

A similar experiment was conducted with tobacco VBI-0 cells and increasing concentrations of cryptogein as an elicitor [18–20] with a very similar outcome (Fig. 2A). Activation of phospholipase A by cryptogein in tobacco cells resulted in an increase of both the pools of fluorescent FA to 156% by 0.5 μM cryptogein and to 268% by 3 μM cryptogein and the fluorescent LPC was increased to 253% by 0.5 μM cryptogein and to 419% by 3 μM cryptogein, respectively. The increase in LPC was stronger and very much consistent with cryptogein as an elicitor than in the parsley system. Again, cryptogein decreased the DAG pool quite clearly to 21% and 27% at 0.5 μM and 3 μM cryptogein, respectively. PA, if present at all, was insubstantial in amount.

As the third stimulus we used the wasp venom tri-decapeptide mastoparan which is thought to mimic the $G\alpha$ -stimulating loop in G-coupled receptors in animal cells and to trigger $G\alpha$ -dependent responses [21,22]. In plants the first effect found for mastoparan was the activation of PLA, measured as LPC accumulation [23,24] but mastoparan also activated PI-PLC [25,26] and PLD in plants [12]. In addition, it was found to uncouple proton gradients in plant membrane vesicles [27]. When the metabolite spectrum of tobacco cells after mastoparan treatment was analyzed changes very similar to those of the two elicitor-induced ones were observed, a strong decrease of fluorescent DAG (to 14% at 10 μM mastoparan) and a weaker increase of fluorescent FA (148% at 10 μM mastoparan) and LPC (307% at 10 μM mastoparan) (Fig. 2B). PA increased relative to the control (compare the stimulation of PLD by mastoparan in [12]) but was a far less abundant metabolite than the other three metabolites. This effect similar to that of elicitors is supported by other results where mastoparan was found to act as an elicitor-like substance [25].

The comparison of elicitors and auxins side by side showed as an important difference that low auxin concentrations did not lead to an accumulation of LPC [5] but elicitors can do so ([6] and this work). Probably, under influence of elicitors the LPC pool was not as tightly down-regulated as it seems to be the case in controls and under the influence of auxins [5] so that, in the presence of elicitor, membrane damage and ion leakage may result. The presence of a fluorescent DAG

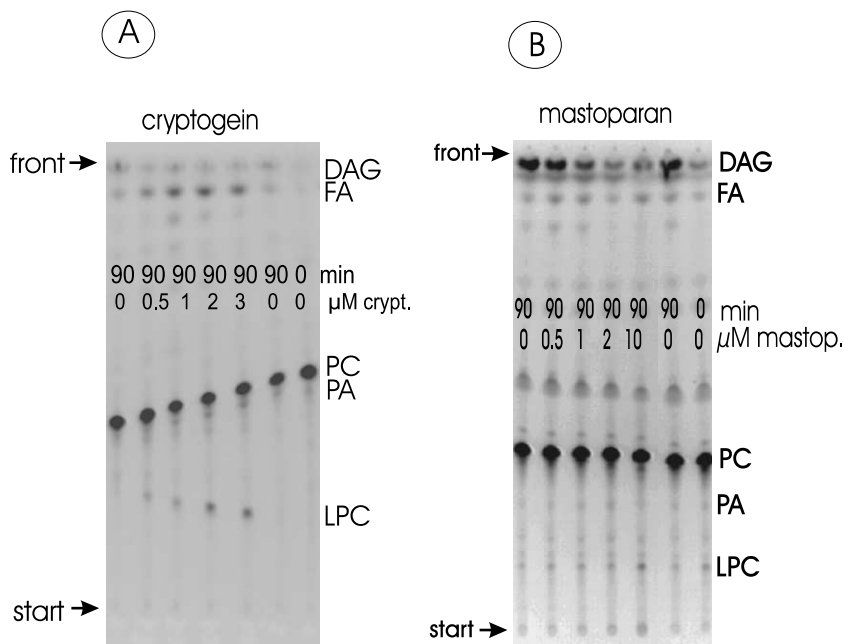


Fig. 2. (A) Thin-layer chromatography of lipids extracted from tobacco cells treated with cryptogein elicitor (0–3 μM) for 90 min. (B) Thin-layer chromatography of lipids extracted from tobacco cells treated with mastoparan (0–10 μM) for 90 min. Solvent system 2 was used for both (A) and (B) and only fluorescent compounds are visible and identified compounds are indicated. DAG = diacylglyceride; FA = free fatty acid; PA = phosphatidic acid; PC = phosphatidylcholine; LPC = lysophosphatidylcholine.

pool but not of a substantial fluorescent PA pool indicated the activity of a PC-hydrolyzing PLC. Alternatively, the consecutive action of PLD and PA phosphatase could have generated this pool of fluorescent DAG. Although this second pathway cannot be totally excluded it seems to be unlikely or minor in contribution so that we conclude that positive evidence for the presence of a PC-PLC was provided. Moreover, we detected five sequences in the *Arabidopsis* genome which are homologous to bacterial PC-PLCs (data not shown). Hence, the elicitors used here probably down-regulated a PC-PLC activity. In plant phospholipid biosynthesis, a PC-PLC has been postulated for the metabolic conversion of phosphatidylethanolamine (PE) to PC, supposed to occur in plants via DAG, on the basis of the fact that both phospholipids had the same fatty acid composition and intermediates were not found [28].

In view of the effects of mastoparan on PC-PLC shown here on PI-PLC [25], PLA [23], and PLD [12] the interpretation for plants of mastoparan action as a substance which mimics a C-terminal loop of G-coupled receptors and activates G α in trimeric G proteins may not hold. In plants only a few G-coupled receptors seem to be present [29,30]. Probably only one type of G α is present in the *Arabidopsis* genome so that the system of G-coupled receptors in plants seems to be more primitive than in animals [31]. It is difficult to perceive how all phospholipases (A, C, and D) stimulated by mastoparan could be coupled to G-coupled receptors [12,23,26]. However, in parsley cells ionophores mimicked the action of the *Phytophthora sojae* elicitor [32] so that the ionophoric property of mastoparan [27] might provide at least an additional explanation for changes in the lipid metabolites observed.

For animals, the existence of a PC-PLC has long been postulated [7], even though no sequence is known. The activation of PC-PLC can be coupled to G-protein-coupled receptors and, recently, it was shown that the $\beta\gamma$ dimer of trimeric G proteins is responsible for the activation of PC-PLC [33]. In addition, tyrosine receptor and ras activation may result in an increase of DAG derived from PC which is sustained and thought to be relevant for long-term growth factor and cell division activity whereas the breakdown of PI lipids by PLC generates a short burst of DAG [34]. Different PKC isoforms respond to DAG pools derived from PI lipids or from PC [35]. Since tyrosine kinase receptors and the ras protein are absent from the *Arabidopsis* genome the receptor linkage of a putative signal-regulated PC-PLC in plants remains an open question.

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