Elicitation of Suspension-Cultured Tomato Cells Triggers the Formation of Phosphatidic Acid and Diacylglycerol Pyrophosphate

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Phosphatidic acid (PA) and its phosphorylated derivative diacylglycerol pyrophosphate (DGPP) are lipid molecules that have been implicated in plant cell signaling. In this study we report the rapid but transient accumulation of PA and DGPP in suspension-cultured tomato (Lycopersicon esculentum) cells treated with the general elicitors, N,N,N,N′-tetraacetyl-chitotetraose, xylanase, and the flagellin-derived peptide flg22. To determine whether PA originated from the activation of phospholipase D or from the phosphorylation of diacylglycerol (DAG) by DAG kinase, a strategy involving differential radiolabeling with [32P]orthophosphate was used. DAG kinase was found to be the dominant producer of PA that was subsequently metabolized to DGPP. A minor but significant role for phospholipase D could only be detected when xylanase was used as elicitor. Since PA formation was correlated with the high turnover of polyphosphoinositides, we hypothesize that elicitor treatment activates phospholipase C to produce DAG, which in turn acts as substrate for DAG kinase. The potential roles of PA and DGPP in plant defense signaling are discussed.

Plants have evolved to defend themselves against pathogenic organisms. The recognition of microorganisms by plants depends on the perception of elicitors generated by the pathogen. Fungal or plant cell wall fragments and molecules secreted by the pathogen induce signaling cascades that activate a cellular response to minimize injury (Dixon et al., 1994; Blumwald et al., 1998). Elicitor perception has been shown to cause changes in the cytosolic calcium concentration (Knight et al., 1991), to induce the oxidative burst (Adam et al., 1989; Mehdy, 1994; Alvarez et al., 1998), to produce nitric oxide (Delledonne et al., 1998; Bolwell, 1999), and to activate MAP-kinase cascades (Ligterink et al., 1997; Stratmann and Ryan, 1998; Zhang et al., 1998; Romeis et al., 1999). The molecular mechanisms underlying these processes and their cooperation in the defense response are subjects of intense study.

Over recent years, a role for phospholipids in plant signal transduction has been recognized. Phosphorylation of inositol lipids by phosphoinositide 3-kinase and the hydrolysis of phospholipids by phospholipases C (PLC), D (PLD), and A2 are thought to produce second messengers that are involved in cell signaling (for review, see Chapman, 1998; Munnik et al., 1998a). A role for phospholipid signaling during the plant cell’s response to elicitors is also emerging (Walton, 1995; Munnik et al., 1998a). The activation of phospholipase A in tomato (Lycopersicon esculentum) leaves (Lee et al., 1997) exposed to pathogens has been shown, as well as its elicitor-induced activation in suspension-cultures of California poppy (Roos et al., 1999) and soybean (Chandra et al., 1996). The free unsaturated fatty acids generated by phospholipase A2 are thought to act as precursors for the synthesis of jasmonic acid, an active inducer of secondary metabolite synthesis in response to pathogen attack (Munnik et al., 1998a). Elicitor treatments are also thought to activate PLC and consequently to change inositol 1,4,5-trisphosphate (IP3) and polyphosphoinositide (PPi) levels in pea epicotyl tissue (Toyoda et al., 1992, 1993) and in cell suspensions of tobacco (Camada and Muto, 1994), soybean (Legendre et al., 1993), and lucerne (Walton et al., 1993). PLC activation has also been demonstrated during the oxidative burst in suspension-cultured soybean cells treated with the elicitor poly-GalUA (Legendre et al., 1993), and the resulting increase in IP3 could explain the ob-

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3 This manuscript is dedicated to the memory of Titus Piatti, who died prior to the publication of this paper.

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served changes in cytosolic calcium that have been measured on treating plants with elicitors (Knight et al., 1991; Mithöfer et al., 1999). Although evidence is mounting for the involvement of PLC, IP₃, and calcium, the function of diacylglycerol (DAG) is still very unclear (Munnik et al., 1998a), although in animal cells it is known to activate several members of the protein kinase C family (Divecha and Irvine, 1995).

A recent study carried out by the Amsterdam lab showed that DAG is rapidly phosphorylated to phosphatidic acid (PA; Munnik et al., 1998b). Stimulation of the green alga Chlamydomonas moewusii with the G-protein activator, mastoparan, induced a transient increase in PA (Munnik et al., 1995), which was due to the hydrolysis of structural phospholipids by phospholipase D (PLD) as well as the combined activities of PLC and DAG kinase (Munnik et al., 1998b).

PA is slowly being recognized as an important lipid second messenger in animal systems. Several proteins, including protein kinases and small G-proteins, are activated by this lipid (for review, see McPhail et al., 1999). Although in plants its function as second messenger still remains to be established, PA is produced by some plant tissues when treated with the plant hormone abscisic acid and, when added in the absence of the hormone, PA mimicked the activity of abscisic acid (Ritchie and Gilroy, 1998; Jacob et al., 1999). In the same way, PA added to C. moewusii cells caused deflagellation, mimicking the effect of mastoparan, that induced its synthesis (Munnik et al., 1995). In recent reports, elevated levels of PA in plants were shown in response to wounding (Lee et al., 1997; Ryu and Wang, 1998), and water stress (Frank et al., 2000).

For any molecule to function as a signal, a down-regulation mechanism should exist to lower the concentration to prestimulation levels. In a recent report, a metabolic derivative of PA was discovered and identified as diacylglycerol pyrophosphate (DGPP; Munnik et al., 1996). This metabolite can be detected during PLC and PLD activation in response to mastoparan, and its formation correlates with the post-stimulation decrease in PA levels (Munnik et al., 1996, 1998b; Van Himbergen et al., 1999). However, to date, it was never recorded in response to a physiological stimulus. Although DGPP formation could be a mechanism to attenuate PA levels, the fact that it is only formed upon cell activation suggests that it could be a signal in its own right.

In the present study, we demonstrate that the levels of PA and DGPP are elevated in tomato cell suspension cultures after treatment with different elicitors. The characterization of these changes in phospholipid metabolism will help to clarify the signaling events that underlie elicitor perception and the induction of plant defense.

**RESULTS**

**32P Incorporation into Tomato Lipids**

To study lipid signaling in tomato, we labeled Msk8 cells with carrier-free orthophosphate (32P) for different time periods. Figure 1A shows the pattern of 32P-labeled phospholipids after separation by thin-layer chromatography (TLC) using an alkaline solvent. With time, [32P] was increasingly incorporated into the structural lipids phosphatidyglycerol, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol (PI) and this continued for up to 24 h (first 4 h are shown in Fig. 1A). In contrast, the minor phospholipids PA, phosphatidylinositol monophosphate (PIP), and phosphatidylinositol bisphosphate (PIP₂) incorporated label relatively faster (Fig. 1B), reaching a maximum after about 60 min. This reflects their labeling via [32P]ATP and

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**Figure 1.** Time course of phospholipid labeling. Suspension-cultured tomato cells were incubated with 32P for the times indicated after which the lipids were extracted and separated by alkaline TLC. A, Autoradiogram of TLC. B, Quantified 32P incorporation into individual lipids expressed as a percentage of the value after 4 h of labeling. Phosphatidyglycerol (PG), ; phosphatidylethanolamine (PE), ; phosphatidylcholine (PC), △; PI, □; PA, ■; DGPP, ○; PIP, ◆; PIP₂, ▲.
their faster turnover, which seems to be typical of signaling lipids (Munnik et al., 1994, 1998a, 1998b). We emphasize this difference in labeling kinetics because we later make use of it to distinguish between PLC and PLD metabolic routes.

Elicitor Treatment Induces the Formation of PA and DGPP

To investigate whether elicitors induce phospholipid signaling, Msk8 cells were incubated with $^{32}$P, for 3 h to label all phospholipids. Cells were then treated with xylanase as an elicitor (Felix et al., 1993) or with cell-free medium as a control. The lipids were extracted and separated by TLC. As shown in Figure 2, treatment with xylanase increased the level of PA starting after 2 min and reaching a maximum after 8 min. In association, the level of PIP$_2$ and, to a lesser extent, PIP declined after 2 min. Shortly after the increase in PA, the level of another minor phospholipid, identified as DGPP, was also seen to increase. DGPP is the phosphorylated product of PA (Munnik et al., 1996).

Figure 3 shows the effect of different concentrations of xylanase on these $^{32}$P-labeled phospholipids. There was a dose-dependent increase in both $[^{32}P]$PA and $[^{32}P]$DGPP after 10 min. The same dose dependence was observed for decreases in the levels of $[^{32}P]$PIP$_2$ (Fig. 3) and $[^{32}P]$PIP (not shown). There was no change in the general kinetics of the responses when different concentrations were used (data not shown).

To see whether these changes are characteristic for elicitor perception in general, we also studied the effects of two unrelated elicitors: the fungal cell wall component chitotetraose ($N,N',N''$-tetraacetylchitosan [CH4]), a tetramer of N-acetyl glucosamine (Côté and Hahn, 1994), and flg22, a peptide of 22 amino acids representing a conserved region in eu-bacterial flagellin, both of which have previously been identified as potent elicitors for Msk8 cells (Felix et al., 1993, 1999). The results of a typical experiment are shown in Figure 4. Clearly, all three elicitors have similar effects, in that they increase the levels of PA and DGPP and decrease the levels of PIP and PIP$_2$, however, the amplitude and the kinetics are different depending on the type of elicitor used. The quickest PA increase was induced by CH4, followed by flg22 and xylanase (Fig. 4A). The increase in PA was coupled to a slower increase in the level of DGPP. The rapid decrease in PIP$_2$ accompanied by a decline in PIP (Fig. 4C and D) suggest that their metabolism could be causally linked to the increase in PA and DGPP.

Distinguishing between PLC- and PLD-Generated PA

The elicitor-dependent increase in PA could occur by phosphorylation of DAG by DAG kinase or by

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Figure 2. Effect of xylanase on phospholipid metabolism. Suspension cultured tomato cells were prelabeled with $^{32}$P, for 3 h and then treated with cell-free medium (A) or with 200 µg mL$^{-1}$ xylanase (B) for the times indicated. Lipids were extracted, separated by TLC and visualized by autoradiography. PA and DGPP are indicated by arrows. A typical result is shown from five independent experiments.

Figure 3. Dose-response effect of xylanase on signaling lipids. $^{32}$P-labeled tomato cells were incubated for 10 min with different concentrations of xylanase. The lipids were then extracted, separated by TLC and the radioactivity quantified by phosphoimaging. Results of a typical experiment ($n = 4$) are shown. PIP$_2$, ■; DGPP, ●; PA, ○.
hydrolysis of structural phospholipids by PLD (Munnik et al., 1998a, 1998b). To distinguish between these pathways, a short labeling strategy was applied, as described earlier in a detailed study using C. moewausii (Munnik et al., 1998b). The method is based on the fact that $^{32}$P is slowly incorporated into structural phospholipids but quickly incorporated into the ATP pool, which is then used to phosphorylate DAG, PA, PI, and PIP to produce their respective $^{32}$P-labeled derivatives (see also Fig. 1). Consequently, a short labeling period more effectively labels these signaling lipids than it labels the structural phospholipids. Accordingly, tomato cells were labeled for just 5 min. As shown in the control of Figure 5 (left panel), while the radioactivity in the structural lipids gradually increased throughout the course of the experiment, that in PA, DGPP, PIP, and PIP$_2$ quickly reached a maximum. When cells were treated with xylanase, the levels of $[^{32}$P]PA and $[^{32}$P]DGPP again increased but now the relative response was even bigger, underlining that they incorporate $^{32}$P from $[^{32}$P]ATP. This suggests that most of the elicitor-induced PA formation is due to the activity of DAG kinase. This response coincided with a decrease in the levels of PIP$_2$ and PIP, putative substrates for PLC.

To test whether PLD also contributed to the elicitor-activated PA formation, the enzyme’s in vivo ability to transfer the phosphatidyl group of its substrate to a primary alcohol was used. The subsequent formation of the product, a phosphatidyl alcohol, is a relative measure of PLD activity (Munnik et al., 1995). PLD activation by elicitors was therefore tested in the presence of 0.8% (v/v) 1-propanol. Accordingly, Msk8 cells were prelabeled with $^{32}$Pi for 3 h and then treated with or without elicitor for 15

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**Figure 4.** The effect of different elicitors on signaling lipids. $^{32}$Pi-Prelabeled Msk8 cells were incubated with cell-free medium or an elicitor for up to 75 min before extracting the lipids and separating them by alkaline TLC. The radioactivity in individual species was quantified and expressed in relation to time zero using the following symbols: control, $\bigcirc$; xylanase, $\square$; CH, $\bullet$; flg22, $\blacksquare$. The lipids, PA, DGPP, PIP, and PIP$_2$ are shown in A, B, C, and D, respectively. Three independent experiments produced similar results, one of which is shown here.

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**Figure 5.** Differential labeling experiment to demonstrate that part of the PA-response is generated through DAG kinase. Suspension-cultured Msk8 cells were incubated for 5 min with $^{32}$P to preferentially label the minor lipids (see Fig. 1). Cells were treated with cell-free medium or xylanase in the presence of excess non-radioactive P$_i$. Lipids were extracted, separated by TLC, and visualized by autoradiography. Results of a representative experiment ($n = 3$) are shown.
Lipids were then extracted and separated using a TLC system that clearly separates phosphatidylpropanol (PPro) from all naturally occurring tomato phospholipids (Munnik et al., 1998b). The radioactivity levels in PA and PPro were quantified by phosphoimaging and represented in a histogram (Fig. 6, A and B).

Despite the consistent activation of PA synthesis by all three elicitors (Fig. 6A), neither CH4 nor flg22 had an effect on the accumulation of P-Pro, indicating that PLD activity could not be detected via transphosphatidylation when stimulated with flg22 and CH4. Moreover, under labeling conditions where PLD-derived PA would be weakly radioactive, elicitor treatment resulted in relatively large amounts of [32P]PA and [32P]DGPP. In support, we invariably detected a decrease in PPIs that coincided with the increase in PA, suggesting that elicitors activated the hydrolysis of PIP2 by PLC to produce DAG, which was subsequently phosphorylated to PA. This is also in line with earlier reports claiming PLC activation in pea, soybean, and lucerne (Toyoda et al., 1992, 1993; Walton et al., 1993; Legendre et al., 1993). The subsequent formation of IP3 could then mediate the rise in cytosolic-calcium concentrations (Knight et al., 1991; Mithofer et al., 1999), although the latter still remains to be shown. Whereas the enhanced levels of DAG are enough to explain the increase in PA, activation of DAG kinase itself cannot be excluded.

Only when xylanase was used as elicitor, a consistent 1.3-fold activation of PLD was detected (Fig. 6). This is in sharp contrast to what was observed after stimulation with the mastoparan analog, mas7, where an approximately 10-fold activation was observed. Plants contain at least three different types of PLD (Pappan and Wang, 1999), therefore mastoparan and xylanase could activate different enzymes producing different quantitative and qualitative effects. Alternatively, if the PLD enzymes have different intracellular localization, as known from both mammalian and plant studies (Xu et al., 1996; Fan et al., 1999; Liscovitch et al., 1999), they could be differentially accessible for the alcohol used to assay PLD’s activity. Another possibility is that PLD could be affected by changes in its molecular environment, for example PIP2 is required for the activity of some PLD isozymes (Chung et al., 1997; Pappan et al., 1997), therefore the metabolism of PIP2 recorded here could account for the lack of PLD activation, in particular since the most dramatic effects on PIP2 levels were observed for CH4 and flg22, which did not activate PLD at all.

**DISCUSSION**

The rapid synthesis of PA and the subsequent formation of DGPP are newly discovered signaling events that take place when tomato cells are treated with elicitors. They are elements in a lipid-signaling pathway that should be considered as part of a network that includes MAP kinases, phosphatases, and calcium-signaling pathways, all involved in the activation of the plant’s defense response.

The production of PA during signaling can result from PLD or DAG kinase activity. The enzyme involved can determine where PA is formed in the cell and what its fatty acid composition is. Both properties could determine which downstream targets are activated (Pettitt et al., 1997). In tomato cells treated with elicitors, the dominant contributor to PA production is DAG kinase, since PLD activity could not be detected via transphosphatidylation when stimulated with flg22 and CH4. Moreover, under labeling conditions where PLD-derived PA would be weakly radioactive, elicitor treatment resulted in relatively large amounts of [32P]PA and [32P]DGPP. In support, we invariably detected a decrease in PPIs that coincided with the increase in PA, suggesting that elicitors activated the hydrolysis of PIP2 by PLC to produce DAG, which was subsequently phosphorylated to PA. This is also in line with earlier reports claiming PLC activation in pea, soybean, and lucerne (Toyoda et al., 1992, 1993; Walton et al., 1993; Legendre et al., 1993). The subsequent formation of IP3 could then mediate the rise in cytosolic-calcium concentrations (Knight et al., 1991; Mithofer et al., 1999), although the latter still remains to be shown. Whereas the enhanced levels of DAG are enough to explain the increase in PA, activation of DAG kinase itself cannot be excluded.

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This is the first time that the formation of DGPP has been studied in detail during signaling in higher plant cells. This phospholipid has recently been identified and characterized in Chlamydomonas spp. (Munnik et al., 1996, 1998b; Van Himbergen et al., 1999). DGPP is barely detectable in non-stimulated cells, even though the enzyme responsible for its synthesis, PA kinase, appeared to be constitutively present in all plants (Wissing and Behrbohm, 1993; Munnik et al., 1998a). This suggests that DGPP formation is strictly coupled to lipid signaling and, as the phosphorylated derivative of PA, is specifically coupled to increases in PA (Munnik et al., 1996). The fact that DGPP is synthesized when the level of PA declines, suggests a role for this lipid in attenuating the PA signal (Munnik et al., 1998a, 1998b). Although DGPP has not yet been identified in animals, it was recently shown to activate a MAP-kinase pathway in macrophages (Balboa et al., 1999). It therefore has the potential to be a signaling molecule in its own right, and should be tested as such in plant systems where its synthesis is correlated with signaling activity.

The different elicitors used in this study have previously been observed to trigger extracellular alkalization with distinct kinetics (Felix et al., 1993, 1999). For CH4, the extracellular pH increased after a lag phase of approximately 0.8 min, for flg22 after 1.5 to 2 min, and for xylanase after 3 to 4 min. As shown in this study, the same elicitors also induced the accumulation of PA with different kinetics, exhibiting similar lag phases. This suggests that they are closely related events, perhaps even causally related. However, it does not explain why different elicitors activate the same responses with different kinetics. A trivial possibility is that the kinetics reflect differences in diffusion rates through the cell wall, since the larger the elicitor, the slower the response. Another explanation is that each elicitor activates specific as well as common signaling pathways that determine the general kinetics. For example, the fact that tomato cells, which are refractory for 8 h to a second dose of CH4, remain responsive to xylanase strongly argues for distinct perception systems and, possibly, distinct pathways that lead to the alkalization response (Felix et al., 1993). In support, xylanase treatment rapidly activated PLD, as shown here, but also activated the transcription of genes coding for phenyl-ammonia-lyase and ACC oxidase, whereas CH4 and flg22 were without effect (Felix et al., 1991, 1993, 1994, 1998; Spanu et al., 1991).

Before PA can be generally accepted as a second messenger in plant cells, specific downstream targets and responses must be identified. So far, PA has been shown to activate deflagellation in Chlamydomonas spp. (Munnik et al., 1995), inhibit α-amylase synthetization in barley aleurone cells (Ritchie and Gilroy, 1998), and activate stomatal closure via inhibition of the inward K+ channel in fava bean leaves (Jacob et al., 1999). It is interesting that a calcium-dependent protein kinase from carrot has recently been found to be activated by PA in vitro (Farmer and Choi, 1999). In animal cells, more putative targets for PA have been identified. These include type I PIP 5-OH-kinase (Moritz et al., 1992; Jenkins et al., 1994), a protein phosphatase (Kishikawa et al., 1999), and a variety of protein kinases (Limatola et al., 1994; Ghosh et al., 1996; Deak et al., 1999; McPhail et al., 1999; Rizzo et al., 1999). Of particular relevance to PA’s potential function in plant defense, is the activation of an NADPH oxidase complex by PA in neutrophils (for review, see McPhail et al., 1999). A PA-dependent protein kinase mediates the functional reconstitution of this complex at the neutrophil plasma membrane (Waite et al., 1997; McPhail et al., 1999). Plant homologs that constitute the NADPH oxidase complex and that are potential targets for this protein have been identified in tomato cells (Xing et al., 1997; Keller et al., 1998), but the kinase and its dependence on PA have yet to be established in plants.

MATERIALS AND METHODS

Materials

Mas7 was purchased from Peninsula Laboratories (Belmont, CA) and xylanase (Trichoderma viride) from Fluka BioChemika (Buchs, Switzerland). Stock solutions of 100 μm mas7, 5 mg mL−1 xylanase, 1 mg mL−1 CH4 (Seikagaku, Tokyo), and 10 mM flg22 (Felix et al., 1999) were made in water and stored at −20°C. Every experiment was performed with a fresh aliquot. Reagents for lipid extraction and subsequent analyses, as well as Silica 60 TLC plates (20 × 20 cm) were purchased from Merck (Darmstadt, Germany).

Cell Cultures

Suspension-cultured tomato (Lycopersicon esculentum Mill.) cells, line Msk8, were grown in Murashige-Skoog liquid medium supplemented with 5.4 μM NAA, 1.0 μM 6-benzyladenine, and vitamins as described by Felix et al. (1991). Cells were continuously rotated at 125 rpm in the dark at 24°C and used 4 to 5 d after subculture.

32P-Phospholipid Labeling and Analyses

Msk8 cells were prelabeled for 3 h with 37-kBq carrier-free 32P (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). They were then treated with xylanase (200 μg mL−1), flg22 (44 ng mL−1), or CH4 (25 ng mL−1) for the times indicated. For control treatments, cell-free medium was used, i.e. conditioned growth medium filtered through a 0.2-μm membrane filter. For pulse-labeling experiments, cells were labeled 5 min with 32P, followed by the addition of 2 mM K2HPO4/KH2PO4 (pH 6.0). Incubations were stopped by withdrawing 170-μL samples, adding them to 20 μL of 50% (v/v) perchloric acid and snap-freezing them in liquid N2. Lipid extraction was initiated by adding 3.75 volumes of CHCl3/methanol:HCl (50:100:1, v/v) and again
snap-freezing in liquid N₂. A two-phase system was induced by the addition of 3.75 volumes of CHCl₃ and 1 volume of 0.9% (w/v) NaCl. Tubes were vigorously shaken for 10 min, centrifuged for 2 min in a microcentrifuge, and their upper-phases removed. The organic phases were washed once with 3.75 volumes of CHCl₃:MeOH:1 M HCl (3:48:47, v/v). Samples were dried by vacuum centrifugation, dissolved in CHCl₃, and analyzed by TLC using an alkaline solvent system (CHCl₃:MeOH:H₂O [60:70:4:16, v/v]) and heat-activated impregnated TLC plates (1.2% [w/v] potassium oxalate, 2 mM EDTA in methanol:H₂O (2:3, v/v), as in Munnik et al. (1994). Lipids were visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA).

In Vivo PLD Measurements

To assay PLD activity in living cells, the production of phosphatidylpropanol (PPro) was measured (Munnik et al., 1995). In brief, cells were prelabeled with ³²P for 16 h and subsequently treated with cell-free medium, mas7, or one of the above-mentioned elicitors, in the presence of 0.8% (v/v) 1-propanol for 15 min. Incubations were stopped and the lipids extracted as described above. [³²P]PPro was separated from the rest of the phospholipids on a heat-activated TLC plate using the organic upper phase of a novel ethyl acetate mixture (Munnik et al., 1998): ethyl acetate:iso-octane:formic acid:water (12:2:3:10, v/v). The detection and quantification of lipids were performed as described above.

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