Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate

Teun Munnik1*, Harold J.G. Meijer1, Bas ter Riet1, Heribert Hirt2, Wolfgang Frank3, Dorothea Bartels4 and Alan Musgrave1
1Institute for Molecular Cell Biology, BioCentrum Amsterdam, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands,
2Vienna Biocenter, Department of Microbiology and Genetics, Vienna, Austria,
3Fraunhofer Institut für Umweltchemie und Ökotoxikologie, Schmallenberg, Germany, and
4Max Planck-Institute für Züchtungsforchung, Cologne, Germany

Received 15 November 1999; revised 10 February 2000; accepted 21 February 2000.
*For correspondence (fax +31 20 5257934; e-mail munnik@bio.uva.nl).

Summary

In mammalian cells, phospholipase D (PLD) and its product phosphatidic acid (PA) are involved in a number of signalling cascades, including cell proliferation, membrane trafficking and defence responses. In plant cells a signalling role for PLD and PA is also emerging. Plants have the extra ability to phosphorylate PA to produce diacylglycerol pyrophosphate (DGPP), a newly discovered phospholipid whose formation attenuates PA levels, but which could itself be a second messenger. Here we report that increases in PA and its conversion to DGPP are common stress responses to water deficit. Increases occur within minutes of treatment and are dependent on the level of stress. Part of the PA produced is due to PLD activity as measured by the in vivo transphosphatidylation of 1-butanol, and part is due to diacylglycerol kinase activity as monitored via 32P-PA formation in a differential labelling protocol. Increases in PA and DGPP are found not only in the green alga Chlamydomonas moewusii and cell-suspension cultures of tomato and alfalfa when subjected to hyperosmotic stress, but also in dehydrated leaves of the resurrection plant Craterostigma plantagineum. These results provide further evidence that PLD and PA play a role in plant signalling, and provide the first demonstration that DGPP is formed during physiological conditions that evoke PA synthesis.

Introduction

Plant cells experience osmotic stress when the solute concentration in their apoplasts changes, and quickly respond with compensatory adaptations to re-establish the osmotic equilibrium. In order to understand osmotic adaptation, components of the stress-induced signal transduction pathways must be identified.

Osmotic stress varies from hypo- to hyperosmotic, and probably activates different receptors depending on the nature and level of stress. This is the case in yeast where the stress receptor Sho1p responds to NaCl concentrations between 200 and 300 mM, while a second receptor, Shn1p, responds to concentrations between 100 and 600 mM (Maeda et al., 1995). Another, as yet unidentified, yeast receptor responds only to NaCl concentrations above 900 mM (Dove et al., 1997). As a consequence of activating different receptors, different signalling pathways will be activated. The osmo-stress pathways identified so far in plants include abscisic acid (ABA, Bonetta and McCourt, 1998), MAP kinases (Hirt, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Munnik et al., 1999), Ca2+ (Bressan et al., 1998), phospholipases (Chapman, 1998; Munnik et al., 1998a), and lipid kinases (Dove et al., 1997; Meijer et al., 1999). The first plant osmosensor has just been identified in Arabidopsis and resembles the Sln1 sensor in yeast (Urao et al., 1999).

Early research on osmo-stress-induced lipid signalling implicated phospholipase C (PLC) activation. The potential signals generated – inositol 1,4,5-trisphosphate (InsP3), diacyl glycerol (DAG) and Ca2+ – are thought to cause cell shrinkage (reviewed by Coté, 1995; Munnik et al., 1998a).
Whether DAG is directly involved in signalling is dubious, because a protein kinase C (PKC) has not been isolated from plants (Munnik et al., 1998a). However, a metabolic product of DAG could be an active signal.

In mammalian cells, DAG is rapidly phosphorylated to phosphatidic acid (PA) by DAG kinase. Originally this was thought to down-regulate DAG levels and to attenuate PKC activation, but currently PA is seen as a novel lipid second messenger (reviewed by English, 1996; McPhail et al., 1999; Munnik et al., 1998a; Topham and Prescott, 1999). Phosphatidic acid targets identified so far include NADPH oxidase (Erickson et al., 1999; Waite et al., 1997), PtdInsP 5-OH-kinase (type I enzymes, Jenkins et al., 1994; Moritz et al., 1992), and a variety of protein kinases such as protein kinase C ζ (Limatola et al., 1994), Raf-1 kinase (Ghosh and Bell, 1997; Ghosh et al., 1996; Rizzo et al., 1999) and novel kinases (reviewed in McPhail et al., 1999). In addition, PA action has been tightly coupled to membrane trafficking, regulation of the cytoskeleton and the oxidative defense reactions in neutrophils (English, 1996; McPhail et al., 1999; Topham and Prescott, 1999).

PA is not only produced indirectly via PLC and DAG kinase, but can be directly generated by phospholipase D (PLD). PLD hydrolyzes structural phospholipids such as phosphatidylcholine and phosphatidylethanolamine and, like PLC, it is activated in response to a variety of signals and is implicated in multiple signalling pathways (Munnik et al., 1998a).

A picture of PLD and PA signalling in plant cells is slowly emerging (reviewed by Munnik et al., 1998a; Wang, 1999). In general, PLD activity is associated with plant stress responses. More specifically, it is thought to play a role in the response to pathogens (Chapman, 1998; Young et al., 1996), wounding (Lee et al., 1997; Ryu and Wang, 1998), water stress (Frank et al. 2000; Munnik et al., unpublished data), and the plant stress hormones ethylene (Fan et al., 1997; Lee et al., 1998) and abscisic acid (Fan et al., 1997; Jacob et al., 1999; Ritchie and Gilroy, 1998). Some of these effects may be mediated by G-protein-coupled stress receptors, because G-protein activators such as mastoparan, alcohols and cholera toxin activate PLD in the absence of stress (De Vrije and Munnik, 1997; Munnik et al., 1995; Munnik et al., 1998b; Van Himbergen et al., 1999). As yet few PA-induced responses have been reported, but treatment of the green alga _Chlamydomonas moewusii_ immediately resulted in deflagellation (Munnik et al., 1995), while similar treatment of barley aleurone cells inhibited the secretion of α-amylase (Ritchie and Gilroy, 1998), and recently PA has been shown to induce stomatal closure (Jacob et al., 1999). In both the latter responses PA mimicked the effects of abscisic acid.

Another potential lipid signal is produced when PA is phosphorylated further to the novel phospholipid diacylglycerol pyrophosphate (DGPP). While this lipid was originally identified as an _in vitro_ product of PA kinase activity (Wissing and Behrbohm, 1993a), we discovered that it was rapidly produced _in vivo_ during G-protein activated signalling (Munnik et al., 1996; Munnik et al., 1998b). As high DGPP levels are rapidly attenuated (Munnik et al., 1998b), it is attractive to think of this lipid as another intracellular signal (Munnik et al., 1996; Munnik et al., 1998a; Wu et al., 1996). This idea was recently supported by demonstrating that DGPP activates a signalling cascade in human macrophages that involves PLA₂, MAP kinases and subclasses of protein kinase C (Balboa et al., 1999). Despite DGPP’s general presence in plants (Munnik et al., 1996; Munnik et al., 1998a; Wissing and Behrbohm, 1993b), yeast (Toke et al., 1998a; Toke et al., 1998b; Wu et al., 1996) and trypanosomes (Marchesini et al., 1998), changes in the level of DGPP under physiological conditions have yet to be established.

In this study we investigated the phospholipid signalling events induced by hyperosmotic stress. We report here that salts, sugars and drought induce immediate activation of PLD and the accumulation of PA and DGPP, suggesting that these phospholipids are involved in signalling water stress in plant cells.

**Results and Discussion**

**NaCl stimulates the formation of PA and DGPP in a time- and dose-dependent manner**

In order to test whether lipid metabolism was affected by hyperosmotic stress, we first prelabelled _Chlamydomonas moewusii_ cells in HMCK medium with ^32_Pi and then added NaCl to produce final concentrations ranging from 50–500 mM. After 5 min lipids were extracted, separated by TLC and the radioactivity in each spot quantified by phosphoimaging. Salt concentrations above 150 mM stimulated the accumulation of PA and DGPP, whereby the amounts of these two phospholipids were proportional to the concentrations of NaCl added, as shown in Figure 1(a,b). The effect of severe treatment was dramatic: within 5 min cells in 500 mM NaCl had increased their PA level sevenfold and their DGPP level 14-fold. The increase in PA appeared to be less because of the higher background levels in non-stimulated cells, as PA is an important intermediate in the biosynthesis of phospholipids, and because DGPP contains two phosphates whereas PA contains only one. However, under the conditions applied the radioactivity in the structural phospholipids did not increase (data not shown). Therefore these results suggest that the accumulation of PA and DGPP is not a general metabolic reaction to salt stress, but may primarily function in signal transduction.

A general property of intracellular signals is that any increase in concentration is transient so that the signal

level can increase again in response to another stimulus. To study the nature of the increase in PA and DGPP, radiolabelled cells were treated with 300 mM NaCl and samples were taken at subsequent intervals to follow changes in phospholipid levels. A typical result is shown in Figure 1(c,d), illustrating the temporary nature of the increase in both lipids, with maxima at about 15 min. As DGPP is synthesized from PA during signalling by the activity of PA-kinase (Munnik et al., 1996), changes in PA levels slightly preceded those in DGPP, while the latter response lasted longer (note log scale).

PA and DGPP elevations are general responses to hyperosmotic stress

Although increasing the external NaCl concentration can represent an osmotic stress, it could simply represent ion toxicity, especially as this alga is cultivated in the absence of NaCl. Therefore other osmoticia were applied for 5 min in concentrations equivalent to 300 mM NaCl. As shown in Table 1, they all activated PA and DGPP synthesis. In separate time-course experiments the kinetics of synthesis were found to be very similar to those observed for 300 mM NaCl (results not shown; Figure 1). Together these data illustrate that accumulation of PA and DGPP is rapidly induced by all osmoticia, and is therefore a general response to hyperosmotic stress in C. moewusii.

### Table 1. Hyperosmotic stress triggers PA and DGPP formation

<table>
<thead>
<tr>
<th>Osmolyte</th>
<th>PA increase</th>
<th>DGPP increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.7 ± 0.2</td>
<td>6.8 ± 4.2</td>
</tr>
<tr>
<td>KCl</td>
<td>3.1 ± 0.2</td>
<td>7.4 ± 4.7</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.6 ± 0.4</td>
<td>8.6 ± 3.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.9 ± 0.1</td>
<td>6.5 ± 2.9</td>
</tr>
<tr>
<td>Mannitol</td>
<td>2.8 ± 1.0</td>
<td>9.2 ± 1.7</td>
</tr>
</tbody>
</table>

32P-prelabelled Chlamydomonas cells were stimulated for 5 min with buffer alone or with 0.55 os kg⁻¹ (equivalent to 300 mM NaCl) of one of the compounds described above. Results are presented as the means of three independent experiments (± SD).

Hyperosmotic stress activates PLD

The synthesis of PA can result from different lipid signalling pathways. PA can be produced either through an increase in DAG kinase activity, for example due to high levels of DAG being formed via the PLC pathway, or through increased PLD activity. As PLD has not yet been involved in signalling hyperosmotic stress, we measured the effect of 300 mM NaCl on in vivo PLD activity by repeating the time-course experiments in the presence of 0.25% 1-butanol. This was done because PLD can transfer the phosphatidyl group of its substrate not only to its natural substrate, water, but also to butanol, producing phosphatidylbutanol (PBut) which can be easily determined as a measure of the in vivo PLD activity (De Vrije and Munnik, 1997; Munnik et al., 1995; Munnik et al., 1998b). It should be noted that this assay cannot determine absolute PLD activities, because most phosphatidyl groups are still transferred to water, forming PA. Figure 2(b) illustrates a typical time-course experiment, with the synthesis of PBut increasing within the first minute of treatment and reaching a maximum at 10 min. The increase in the synthesis of PBut was always coupled to a larger increase in PA (Figure 2a), establishing that PLD is rapidly activated by high concentrations of NaCl as well as by other osmoticia (data not shown).

Part of the PA response is due to phosphorylation of DAG

The formation of PBut provides a relative measure of PLD activity, yet the increase after a 10 min treatment with 300 mM NaCl was about 2.5-fold (Figure 2b), whereas the increase in PA was about eightfold (Figure 2a), suggesting that PLD activity was not the only contributor to PA formation. To investigate the possible contribution of DAG...
kinase-generated PA, a differential labelling technique (Munnik et al., 1998b) was used. In short, *Chlamydomonas* cells were prelabelled with $^{32}$P$_i$ for 5 min before treating them with a range of NaCl concentrations. Under such short labelling conditions, the structural lipids that are hydrolysed by PLD are hardly labelled, and consequently the PA generated by PLD is hardly labelled. In contrast, lipids synthesized by the incorporation of $^{32}$P from ATP, for example PA synthesized by DAG kinase, are strongly labelled. Therefore any increase in $^{32}$P-PA synthesis under these conditions cannot be due to PLD but is due to DAG kinase (for a detailed validation of this technique see Munnik et al., 1998b).

To test whether $^{32}$P-PA was formed under these circumstances, cells were treated for 5 min with NaCl concentrations ranging from 100 to 400 mM. As shown in Figure 3, treatment again stimulated $^{32}$P-PA synthesis in a dose-dependent manner, indicating that DAG kinase also makes a strong contribution to PA synthesis during osmotic stress. The simplest explanation for this increase is that DAG production was increased by PLC activity; we have previously shown that when PLC hydrolyses phosphatidylinositol bisphosphate the DAG generated is rapidly phosphorylated to PA (Munnik et al., 1998b). This is the most likely explanation: a number of reports have shown that hyperosmotic stress activates polyphosphoinositide metabolism (Cho et al., 1993; Heilmann et al., 1999), an increase in InsP$_3$ (Cost, 1995; Heilmann et al., 1999; Smolenska-Sym and Kaperska, 1996), and an increase in intracellular Ca$^{2+}$ (Bressan et al., 1998; Knight et al., 1997). Because vacuolar Ca$^{2+}$ stores become more sensitive to InsP$_3$ after osmo-stress (Allen and Sanders, 1994), and the expression of genes involved in PLC signalling is also increased after water stress (Hirayama et al., 1995; Katagiri et al., 1996; Mikami et al., 1998), it is likely that a PLC pathway is involved in osmo-signalling in plants and *Chlamydomonas*. Nonetheless, we cannot exclude the possibility that part of the response is due to direct activation of DAG kinase.

**Activation of PLD and elevation of PA-and DGPP levels are common plant responses to water stress**

As osmotic stress consistently stimulated PLD activity and the formation of PA and DGPP in *Chlamydomonas*, the question remained as to whether this was a common plant response to hyperosmotic conditions. Therefore tomato and alfalfa cell-suspension cultures were tested. Cell suspensions were chosen rather than intact tissues because they take up $^{32}$P$_i$ and react to treatment more synchronously. Accordingly, cells were prelabelled for 3 h with $^{32}$P$_i$ before treating them for 5 min with different NaCl concentrations in the presence of 0.25% 1-butanol. After extracting and separating the lipids, the radioactivity in the appropriate spots was quantified. As shown in Figure 4, alfalfa and tomato cells also respond to NaCl treatment by increasing the synthesis of PA and DGPP in a dose-dependent manner, and part of the response is again due to PLD activity, as witnessed by the formation of PBut. We therefore conclude that the response is typical of many plant cells.

Although it is convenient to add solutions of osmotica to cell cultures, in nature plants frequently experience osmotic stress under drought conditions. To test whether dehydration activates similar changes in lipid metabolism, we used leaf discs of the dessication-tolerant resurrection plant *Craterostigma plantagineum*. They were prelabelled by floating them for 16 h on a $^{32}$P$_i$-containing solution and exposed to dehydration by transferring to dry filter paper in a fume cupboard for 30 min. As shown in Figure 5, drought stress dramatically increased the levels of PA and DGPP. Part of the PA increase was due to the activation of PLD, because a similar increase in PBut formation was found. A more detailed analysis of PLD activation during drought in *C. plantagineum* is presented by Frank et al. (2000), but the data shown here illustrate that the activation of PLD and the production of PA and DGPP are typical not only for cell cultures under artificial conditions, but also for plant tissues under more natural forms of osmotic stress.
In conclusion, PLD is one of the signalling enzymes that is rapidly activated by hyperosmotic stress. In support of its potential importance to osmo-signalling, a PLD gene has recently been shown to be one of the first expressed in resurrection plants subjected to drought (Frank et al., 2000). This response is similar to the osmo-stress-induced expression of genes involved in PLC and MAP kinase signalling in Arabidopsis and alfalfa (Hirt, 1997; Munnik et al., 1999; Shinozaki and Yamaguchi-Shinozaki, 1997), two other pathways thought to be involved in osmo-signalling. Thus PLD signalling could be subject to positive feedback regulation in order to prime cells for further osmo-stress. In this way plants could adapt to stress, making them more tolerant (Knight et al., 1998). Within the lipid signalling pathways activated by osmotic stress, PA and its phosphorylated derivative DGPP could be key players. Future studies will reveal whether these phospholipids regulate the expression of osmo-stress-inducible genes, or whether they serve other functions.

**Experimental procedures**

**Materials**

Silica 60 TLC plates and reagents for lipid extraction and analyses were from Merck (Darmstadt, Germany). \(^{32}P\)-Orthophosphate (carrier-free) was from Amersham International (Hertogenbosch, The Netherlands). Radioactive DGPP was prepared and identified as described by Munnik et al. (1996).

**Plant material, metabolic radiolabeling and osmo-stress**

Chlamydomonas cells (C. moewusii strain UTEX 10) were cultivated as described earlier (Munnik et al., 1994) and suspended in HMCK buffer (10 mM HEPES pH 7.4 [KOH], 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 mM KCl) at a final concentration of 1–2 \( \times 10^7 \) cells per ml.

Phospholipids were metabolically labelled by incubating cells (1–2 \( \times 10^7 \) cells per ml) with 100 \( \mu \)Ci carrier-free PO\(_4\)\(^{3-} \) per ml in HMCK, usually for 2–4 h but, in a short-term labelling protocol, for
only 5 min (Figure 3; Munnik et al., 1998b). Routinely, 100 µl cell suspension was treated in a total volume of 200 µl for the time and at the stress levels indicated. Incubations were stopped by adding 3.75 vol CHCl₃/MeOH/HCl (50:100:1, by vol), and lipids were extracted, separated and quantified as described earlier (Munnik et al., 1996).

Suspension-cultured alfalfa cells (Medicago sativa ssp. varia cv Rambler, line A2) were cultivated at 24°C in MS medium supplemented with 30 g l⁻¹ sucrose, 1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid and 0.1 mg l⁻¹ kinetin (Munnik et al., 1999). Tomato suspension cells (Lycopersicon esculentum cv. Moneymaker) were grown in the same medium supplemented with B₃ vitamins (Munnik et al., 1996).

Higher plant cell suspensions were grown on a rotary shaker (125 r.p.m.) at 24°C in the dark. Cells were subcultured at intervals of 1 week and used 3–5 days after transfer. They were labelled at room temperature in their own medium using 100 µCi ³²P ml⁻¹ for the times indicated. Cells were osmotically stressed by adding 90 µl of a 2× concentrated solution to an equal volume of cells. Incubations were stopped by adding 20 µl 50% (w/v) perchloric acid and freezing them into liquid nitrogen. Lipids were extracted by adding 3.75 vol CHCl₃/MeOH/HCl (50:100:1 v/v) and further processed as described (Munnik et al., 1996).

Leaf discs of the resurrection plant Craterostigma plantagineum were ³²P-prelabelled and exposed to drought stress as described in detail by Frank et al. (2000).

**PA and DGPP analysis**

Lipids were separated by TLC using an alkaline solvent system (CHCl₃/MeOH/H₂O: 25% NH₄OH/H₂O: 90:70:4:16 by vol; Munnik et al., 1994). Radiolabelled phospholipids were detected by autoradiography and quantified by phosphoimaging (Storm, Molecular Dynamics, Sunnyvale, CA). Identities of DGPP and PA were confirmed by de-acylation and head-group analysis as described by Munnik et al. (1996). Non-radioactive phospholipid standards (approximately 10 µg) were visualized by exposure to iodine vapour.

**PLD measurements**

PLD activity was measured as the production of PBut in living cells essentially as described by Munnik et al. (1995). Briefly, ³²P-prelabelled cells were treated in the presence of 0.25% n-butanol. At subsequent times incubations were stopped and the lipids extracted as described above. The ³²P-labelled PBut was separated from the rest of the phospholipids by a modified ethyl acetate/iso-octane/HCOOH/water 12:2:3:10 by vol; Munnik et al., 1998b). Radioactivity was visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA).

**Acknowledgements**

TM is funded by The Netherlands Organisation for Scientific Research (NWO-PULS; 805-48-005). This work was co-supported by an EMBO fellowship awarded to T.M. We are grateful to our colleagues in the laboratory for helpful discussions, and to Arnold van der Luit, Aveline van Doorn and Claudia Jonak in particular for the tomato and alfalfa suspension cultures.

**References**


Note added in proof

While this paper was in review, a paper by Weigert et al. (1999) was published describing another interesting route of PA synthesis that could be important for signalling, namely by acylation of lysophosphatidic acid. However, we have found no evidence that would support such a pathway in response to osmotic stress.