Osmotically Induced Cell Swelling versus Cell Shrinking Elicits Specific Changes in Phospholipid Signals in Tobacco Pollen Tubes[1]

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Pollen tube cell volume changes rapidly in response to perturbation of the extracellular osmotic potential. This report shows that specific phospholipid signals are differentially stimulated or attenuated during osmotic perturbations. Hypo-osmotic stress induces rapid increases in phosphatidic acid (PA). This response occurs starting at the addition of 25% (v/v) water to the pollen tube cultures and peaks at 100% (v/v) water. Increased levels of PA were detected within 30 s and reached maximum by 15 to 30 min after treatment. The pollen tube apical region undergoes a 46% increase in cell volume after addition of 100% water (v/v), and there is an average 7-fold increase in PA. This PA increase appears to be generated by phospholipase D because concurrent transphosphatidylation of *n*-butanol results in an average 8-fold increase in phosphatidylbutanol. Hypo-osmotic stress also induces an average 2-fold decrease in phosphatidylinositol phosphate; however, there are no detectable changes in the levels of phosphatidylinositol bisphosphates. In contrast, salt-induced hyperosmotic stress from 50 to 400 mM NaCl inhibits phospholipase D activity, reduces the levels of PA, and induces increases in the levels of phosphatidylinositol bisphosphate isomers. The pollen tube apical region undergoes a 41% decrease in cell volume at 400 mM NaCl, and there is an average 2-fold increase in phosphatidylinositol 3,5-bisphosphate and 1.4-fold increase in phosphatidylinositol 4,5-bisphosphate. The phosphatidylinositol 3,5-bisphosphate increase is detected within 30 s and reaches maximum by 15 to 30 min after treatment. In summary, these results demonstrate that hypo-osmotic versus hyperosmotic perturbation and the resultant cell swelling or shrinking differentially activate specific phospholipid signaling pathways in tobacco (*Nicotiana tabacum*) pollen tubes.

The regulation of cellular osmotic pressure is important for metabolism, development, and growth. Plant cells have evolved several mechanisms to respond to changes in the extracellular osmotic potential and to normalize the intracellular pressure or adjust the cytochemistry in response to these changes. Sudden shifts of extracellular osmotic gradients induce dynamic changes in ion fluxes across the plasma membrane as an early osmoregulatory response (Schroeder and Hagiwara, 1989; Schroeder and Hedrich, 1989; Ward et al., 1995; Teodoro et al., 1998; Liu and Luan, 1998; Barbier-Brygoo et al., 2000; Blatt, 2000; Shabala et al., 2000; Ivashikina et al., 2001; Schroeder et al., 2001; L. Zonia, personal observation). Osmoregulatory ion fluxes are also regulated by specific inositol polyphosphate signals (Blatt et al., 1990; Gilroy et al., 1990; Lemtiri-Chlieh et al., 2000; Zonia et al., 2002) and by PI(4,5)P_2-dependent phospholipase C (PLC) signaling (Staxen et al., 1999; Drøbak and Watkins, 2000; DeWald et al., 2001; Munnik and Meijer, 2001; Takahashi et al., 2001). In fact, several phospholipid signals are rapidly activated by osmotic stress (see below). Specific mitogen-activated protein kinase cascades also are activated within minutes after osmotic stress (Cazale et al., 1999; Munnik et al., 1999; Felix et al., 2000; Munnik and Meijer, 2001). Later responses to osmotic perturbation include the production of phytohormones (Assmann and Wang, 2001; Schroeder et al., 2001; Seo and Koshiba, 2002), the accumulation and partitioning of specific solutes (Hare et al., 1998, 1999; Hasegawa et al., 2000; Lefevre et al., 2001; Raymond and Smirnoff, 2002), and the induction of gene expression (Zhu et al., 1998; Hasegawa et al., 2000; Zhu, 2000).

Phospholipid signaling is an important component of the early response to hyperosmotic stress (for review, see Munnik and Meijer, 2001). PI(4,5)P_2 and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] levels increase on hyperosmotic challenge (Heilmann et al., 1999, 2001; Pical et al., 1999; Drøbak and Watkins, 2000; DeWald et al., 2001). Hyperosmotic stress induces the phosphorylation of phosphatidylinositol 3-phosphate (PI3P) to phosphatidylinositol 3,5-

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bisphosphate ($PI(3,5)P_2$) (Meijer et al., 1999). Recent work has demonstrated that PI5P levels increase after salt stress (Meijer et al., 2001b). Hyperosmotic stress activates phospholipase D (PLD) activity and induces increased levels of phosphatidic acid (PA; Frank et al., 2000; Munnik et al., 2000; Katagiri et al., 2001; Munnik, 2001; Meijer et al., 2002). In addition, PLD activity is rapidly activated by abscisic acid, a phytohormone involved in the plant cell's response to desiccation (Ritchie and Gilroy, 1998, 2000; Jacob et al., 1999; Sang et al., 2001; Hallouin et al., 2002). Salt-induced hyperosmotic stress activates phosphalipase A_2 (PLA_2) and increases lyso-PA (Meijer et al., 2001a). Significantly, all previous reports of phospholipid signaling in response to NaCl-induced hyperosmotic stress in other systems (cell suspensions of tomato [*Lycopersicon esculentum*], alfalfa [*Medicago sativa*], Arabidopsis, and *Chlamydomonas*) have shown that the responses are because of hyperosmotic stress and not sodium toxicity (Pical et al., 1999; Munnik et al., 2000; Meijer et al., 2001a, 2001b, 2002; Munnik and Meijer, 2001). In summary, these studies demonstrate that a number of phospholipid signals are induced by hyperosmotic stress. Cellular targets for some of these signals include protein and lipid kinases, components involved in membrane trafficking, and ion channels, and work is currently under way to identify additional targets and define the mechanisms of interaction (for review, see Munnik, 2001; Meijer and Munnik, 2003).

Previous studies of phospholipid signaling in pollen tubes have focused on $PI(4,5)P_2$. A phosphatidylinositol (PI)-specific PLC activity has been identified in pollen tubes, and PLC hydrolysis of $PI(4,5)P_2$ has been demonstrated (Franklin-Tong et al., 1996). In addition, photolysis of caged $Ins(1,4,5)P_3$ injected into pollen tubes has been shown to cause an increase in free Ca^{2+}, with the greatest increase observed in the region of the vegetative nucleus (Franklin-Tong et al., 1996; Malhó, 1998). Further studies have demonstrated a role for $PI(4,5)P_2$ in conjuction with the small G-protein Rac at the pollen tube tip during cell elongation (Kost et al., 1999). Recent work using isolated extracts of pollen tubes has reported the presence of $PI(4,5)P_2$-dependent and -independent PLD activities (Potocky et al., 2003).

Pollen tubes appear to utilize a strategy of controlled hydrodynamics as part of the mechanics that drive cell elongation (Zonia et al., 2001, 2002; L. Zonia personal observations). Pollen germination on the stigmatic surface is dependent on controlled hydration and metabolic activation for the proper initiation of tube growth. Pollen tubes are rapidly growing cells under high turgor pressure and, therefore, must have mechanisms to regulate the internal hydrostatic pressure to avoid cell bursting without compromising growth. The present studies were undertaken to investigate if pollen tubes respond to osmotic perturbation and the resultant cell volume changes by activation of phospholipid signaling pathways. The results show that several phospholipid signals are detected during pollen tube growth, including $PI(3,5)P_2$, $PI(4,5)P_2$, phosphatidylinositol phosphate (PIP), and PA. These signals are differentially induced or attenuated by hypo-osmotic versus salt-induced hyperosmotic stress. Hypo-osmotic stress induces a rapid increase in PA and a decrease in PIP. In contrast, salt-induced hyperosmotic stress induces increases in $PI(3,5)P_2$ and $PI(4,5)P_2$ and decreases the level of PA. The fact that these signals are present during normal growth suggests that mechanisms controlling osmotic status and cell volume are normal components of the biomechanical networks driving pollen tube cell elongation.

RESULTS

Pollen Tube Apical Region Swells or Shrinks in Response to Hypo-Osmotic versus Hyperosmotic Stress

Pollen tube turgor pressure or cell volume is exquisitely sensitive to decreases in the extracellular osmotic potential caused by the addition of water to the culture medium of growing pollen tubes. Germination medium has an osmolarity of 0.36 Osm. A 50% (v/v) water stress treatment is a 2:1 (v/v) culture: water dilution with a decrease in medium osmolarity to 0.24 Osm; a 100% (v/v) water stress treatment is a 1:1 (v/v) culture:water dilution with a decrease in medium osmolarity to 0.18 Osm. Hypo-osmotic stress induces rapid increases in the pollen tube apical cell volume (Fig. 1). The response displays a hyperbolic response curve from 2.5% to 100% (v/v) water (Fig. 1). The apical 50-μm length of untreated tobacco (*Nicotiana tabacum*) pollen tubes has a cell

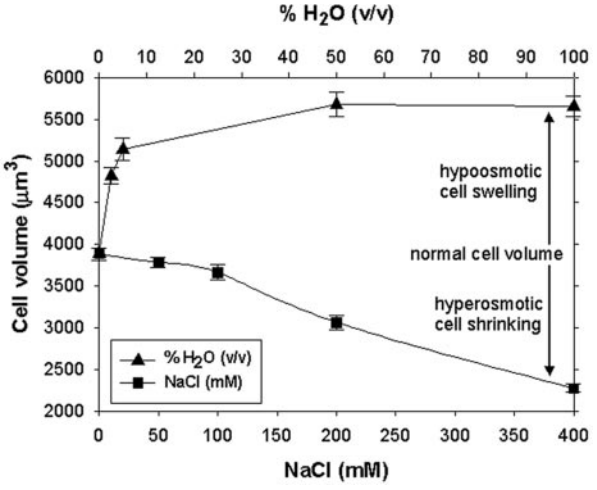

Figure 1. Changes in the apical cell volume of pollen tubes in response to osmotic perturbation. Each value is the mean of 50 pollen tubes ± SE. The region analyzed spans the apical 50-μm length of the pollen tubes. Hypo-osmotic stress is induced by the addition of water and causes cell swelling. Hyperosmotic stress is induced by the addition of NaCl and causes cell shrinking.

volume of 3,884 ± 74 μm^3 (Fig. 1). This increases to 4,822 ± 95 μm^3 after addition of 2.5% (v/v) water (v/v), which is a 24% increase compared with controls (Fig. 1). Cell volume increases to a level of 5,676 ± 144 μm^3 after addition of 50% (v/v) water (v/v), which is a 46% increase compared with controls (Fig. 1). The cell volume is essentially unchanged with hypo-osmotic treatment from 50% to 100% (v/v) water (Fig. 1). Tobacco pollen tubes burst when the apical cell volume increases by approximately 58% compared with normal (Zonia et al., 2002).

Pollen tube turgor pressure is also sensitive to increases in the extracellular osmotic potential caused by the addition of salt to the culture medium of growing pollen tubes. Salt-induced hyperosmotic stress induces decreases in the pollen tube apical cell volume (Fig. 1). The cell volume response displays a linear decrease from 100 to 400 mM NaCl (Fig. 1). These stress treatments correspond to an increase in the medium osmolarity from 0.36 to 0.56 and 1.16 Osm for 100 and 400 mM NaCl, respectively. The cells begin to plasmolyze at the apex and in the apical region at 200 mM NaCl, and the apical cell volume decreases to 3,064 ± 85 μm^3, which is a 21% decrease compared with controls (Fig. 1). At 400 mM NaCl, the cells undergo severe plasmolysis at the apex, and the apical cell volume decreases to 2,274 ± 48 μm^3, which is a 41% decrease compared with controls (Fig. 1). Cytoplasmic streaming still occurs at a slow rate, indicating that the cells are still viable when subjected to these high salt concentrations (data not shown).

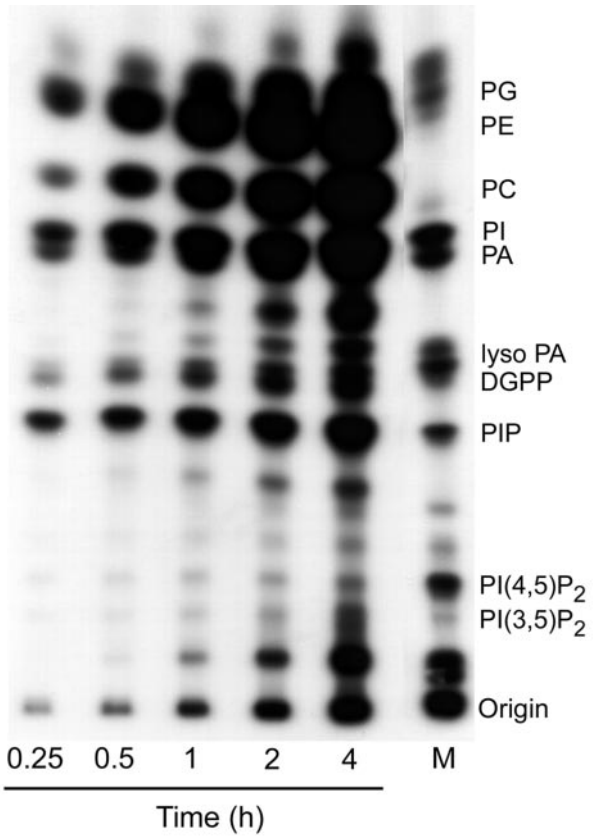

Figure 2. Identification of phospholipids during pollen tube growth. $^{32}P_i$ was added to pollen tube cultures, and lipids were extracted at the times indicated and analyzed by TLC using an alkaline solvent system. Lane 1, 15 min. Lane 2, 30 min. Lane 3, 1 h. Lane 4, 2 h. Lane 5, 4 h. Lane 6, Phospholipid marker standard.

Several Different Signaling Phospholipids Are Present during Pollen Tube Growth

The relative turnover rates and abundances of phospholipid signals present during normal pollen tube growth are determined by time course studies of the incorporation of label into phospholipids. For these studies to establish incorporation rates and phospholipid abundances, pollen are germinated and allowed to grow for 4 h before adding $^{32}PO_4^{3-}$ ($^{32}P_i$) to the cultures (100 μCi ml^{-1}). Phospholipids are extracted during time course studies and analyzed by thin-layer chromatography (TLC) using an alkaline solvent system (note that this time course method of labeling is not the method routinely used for stress studies, which is described in "Materials and Methods"). Pollen tubes rapidly incorporate $^{32}P_i$ into both signaling and structural phospholipids (Fig. 2). Within 15 min (the earliest time point tested; Fig. 2, lane 1), phospholipids involved in signaling pathways can be detected, including PI(3,5)P_2, PI(4,5)P_2, PIP, PA, diacylglycerol (DAG) pyrophosphate, and PI, in addition to the structural phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). After 4 h of labeling, all phospholipids incorporate a high level of $^{32}P_i$ (Fig. 2, lane 5). The relative rates of $^{32}P_i$ incorporation during the first 15 min are highest for PI(3,5)P_2, PI(4,5)P_2, PIP, PA, and PI, suggesting their high rate of turnover (Fig. 3, A–E). The structural phospholipids PC, PE, and PG have higher rates of $^{32}P_i$ incorporation after an initial time lag (Fig. 3, F–H). After labeling for 4 h, the percentage of $^{32}P_i$ incorporated by phospholipids involved in signaling pathways (with respect to total phospholipid $^{32}P_i$) is 0.097% PI(3,5)P_2, 0.061% PI(4,5)P_2, 1.48% PIP, 4.87% PA, and 13.04% PI (Fig. 3). These results demonstrate that several signaling phospholipids are present during normal pollen tube growth.

PLD Activity Is Constitutive during Pollen Tube Growth

The observation of relatively high PA and DAG pyrophosphate levels in untreated pollen tubes suggests that PLD may be active during normal growth (Fig. 2). This is assessed by the addition of *n*-butanol to pollen tube cultures. *n*-Butanol is a competitive substrate for the transphosphatidylation activity of PLD; therefore, phosphatidylbutanol (PBut) serves as

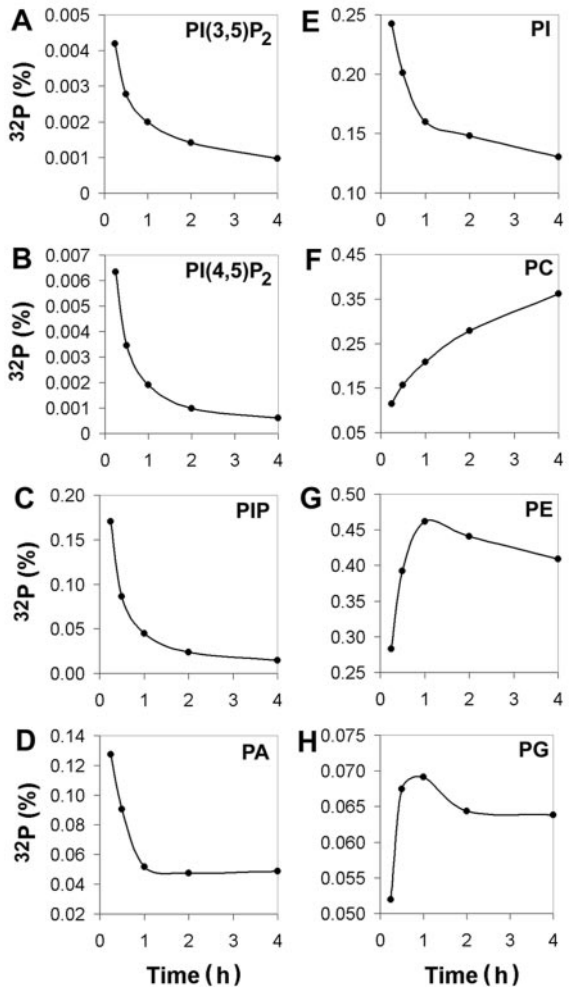

Figure 3. Rate of incorporation of $^{32}P_i$ into pollen tube phospholipids. Data are expressed as percentage of $^{32}P_i$ incorporated into each phospholipid with respect to total $^{32}P_i$ incorporated into all phospholipids at each time point. Phospholipids with high turnover rates have high initial rates of $^{32}P_i$ incorporation, whereas structural phospholipids have higher incorporation of $^{32}P_i$ after an initial time lag. A, $PI(3,5)P_2$. B, $PI(4,5)P_2$. C, PIP. D, PA. E, PI. F, PC. G, PE. H, PG.

a useful marker of in vivo PLD activity (Munnik et al., 1995; Munnik, 2001). Phospholipids are analyzed during time course studies after the addition of 0.1% (v/v) *n*-butanol and identified by TLC using an ethyl acetate solvent system. PA is detected in untreated pollen tubes (Fig. 4A, lane 1), and PBut can be detected within 5 min after the addition of *n*-butanol (the earliest time point tested; Fig. 4A, lane 2). There is a steady increase in the level of PBut during 60 min after the addition of 0.1% (v/v) *n*-butanol (the latest time point tested; Fig. 4A, lanes 2–5). PA has a high rate of turnover (Fig. 3D), and PA levels increase slightly during growth (Fig. 4B). However, PBut accumulates to higher levels than PA because PBut is not a natural metabolite (Fig. 4B). These results indicate that PLD activity is constitutive during normal growth of pollen tubes.

Hypo-Osmotic Stress Stimulates PLD Activity, Generates a Large Increase in PA, and Reduces PIP

Phospholipids are analyzed during hypo-osmotic perturbation studies to investigate if specific signals are induced. The most stunning response is a large increase in the level of PA (Fig. 5A). PA levels increase significantly starting at 25% (v/v) water and reach maximum at 50% to 100% (v/v) water (Fig. 5, A and B). The levels of PA and PBut increase with similar kinetics during 10 min after treatment with increasing hypo-osmotic stress (Fig. 5B). PA levels increase within 30 s after 100% (v/v) water and reach an average 6.6-fold increase within 15 to 30 min after treatment (Fig. 6A). PBut levels reach an average 8-fold increase within 30 min after 100% (v/v) water (Fig. 6B). The observation of similar kinetics in increases in PA and PBut during dose response (Fig. 5) and time course (Fig. 6) studies suggests that most PA induced by hypo-osmotic stress is generated through the PLD pathway.

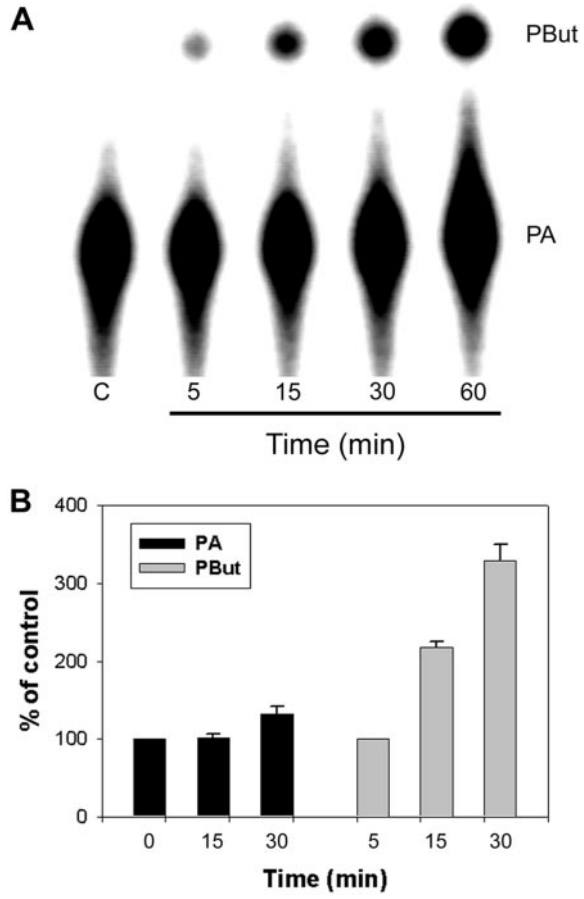

Figure 4. Analysis of PLD activity during pollen tube growth. A, Lipids were extracted and analyzed by TLC using an ethyl acetate solvent system. Lane 1, Control in the absence of *n*-butanol. Lanes 2 to 5, 5, 15, 30, and 60 min after the addition of 0.1% (v/v) *n*-butanol. B, Increasing accumulation of PA and PBut during pollen tube growth indicates constitutive activity of PLD. PBut values are expressed as percentage increase with respect to the 5-min sample.

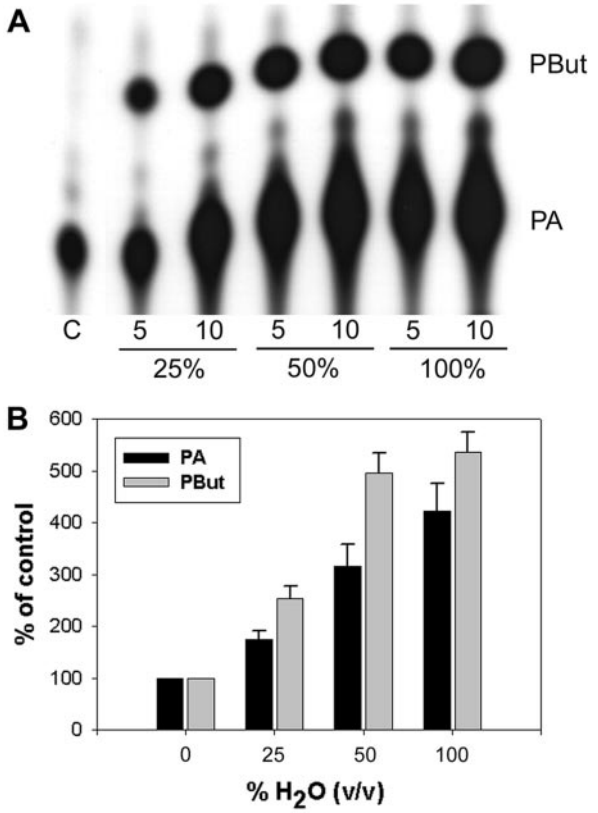

Figure 5. Hypo-osmotic stress stimulates PLD activity and generates increased levels of PA. Lipids were extracted and analyzed by TLC using an ethyl acetate solvent system. A, Lane 1, control in the absence of *n*-butanol. Lanes 2 and 3, 5 and 10 min after the addition of 25% (v/v) water. Lanes 4 and 5, 5 and 10 min after the addition of 50% (v/v) water. Lanes 6 and 7, 5 and 10 min after the addition of 100% (v/v) water. B, Concentration-dependent increases in PA and PBut with increasing hypo-osmotic stress (percentage water, v/v). All samples analyzed at 10 min after the start of hypo-osmotic stress.

Hypo-osmotic stress also induces a significant decrease in the level of PIP. The greatest decrease is observed after 100% (v/v) water, although reduced levels can also be detected after 50% (v/v) water (Fig. 7A). At 100% (v/v) water, PIP levels decrease within 2 min after treatment, and by 15 to 30 min, there is an average 2-fold reduction in the level of PIP (Fig. 7, B and C). Significantly, no changes in the levels of phosphatidylinositol bisphosphate (PIP_2) isomers can be detected after hypo-osmotic stress. These results suggest that PI kinase activity may be specifically inhibited or that specific hydrolysis of PIP may occur during the hypo-osmotic stress response.

Significant changes in the levels of the structural phospholipids PC, PE, and PG could not be detected in response to hypo-osmotic stress (data not shown).

Hyperosmotic Stress Attenuates PLD Activity and Induces Increases in $PI(3,5)P_2$ and $PI(4,5)P_2$

Phospholipids are also analyzed during salt-induced hyperosmotic stress studies. As shown in Figure 8A, salt-induced hyperosmotic stress induces a rapid attenuation of PLD activity and decrease in the levels of PA. Normal constitutive PLD activity appears to be inhibited by hyperosmotic stress of 50 to 400 mM NaCl in that no PBut can be detected under these conditions (Fig. 8A). PA levels are reduced by 25% within 30 min after treatment with 400 mM NaCl (Fig. 8B).

Salt-induced hyperosmotic stress and cell volume decrease induce increases in the levels of $PI(3,5)P_2$ and $PI(4,5)P_2$. Small increases in both PIP_2 isomers occur at 100 to 200 mM NaCl, but the largest increases occur at 400 mM NaCl (the highest concentration tested), with an average 2-fold increase in the level of $PI(3,5)P_2$ (Student's *t* test; $P = 0.036$) and an average 1.4-fold increase in the level of $PI(4,5)P_2$ (Student's *t* test; $P = 0.021$) at 30 min after treatment (Fig. 9A). Increases in $PI(3,5)P_2$ are detected within 30 s after the addition of 400 mM NaCl (Fig. 9, B, lane 2, and C) and reach maximum levels within 15 to 30 min (Fig. 9, B, lanes 7 and 8, and C). $PI(4,5)P_2$ levels increase with similar kinetics (Fig. 9, B and C). Structural verification of $PI(3,5)P_2$ was determined by performing mono-methylamine deacylation and head group analysis as described previously in detail (Meijer et

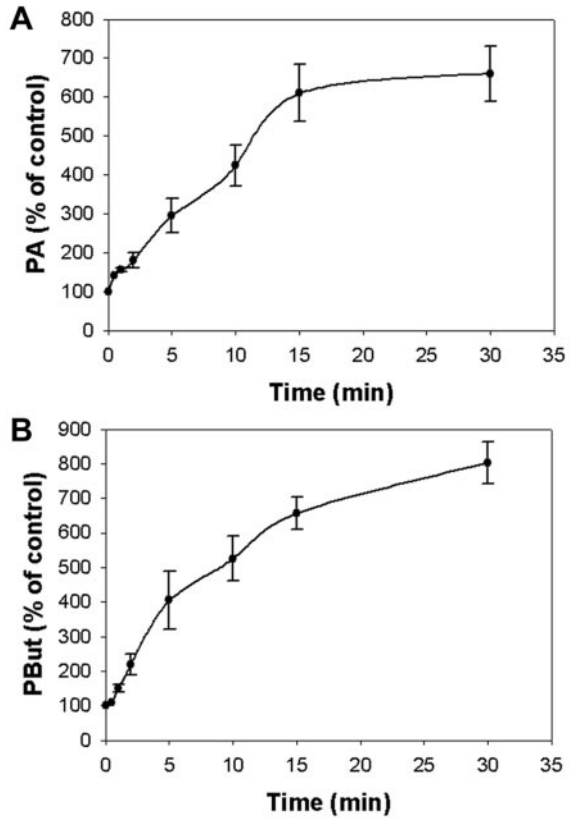

Figure 6. Time-dependent increases in PA and PBut after hypo-osmotic treatment of 100% (v/v) water. Values represent percentage increase of PA compared with untreated control and PBut compared with 10-min sample. A, time course of PA increase. B, Time course of PBut increase.

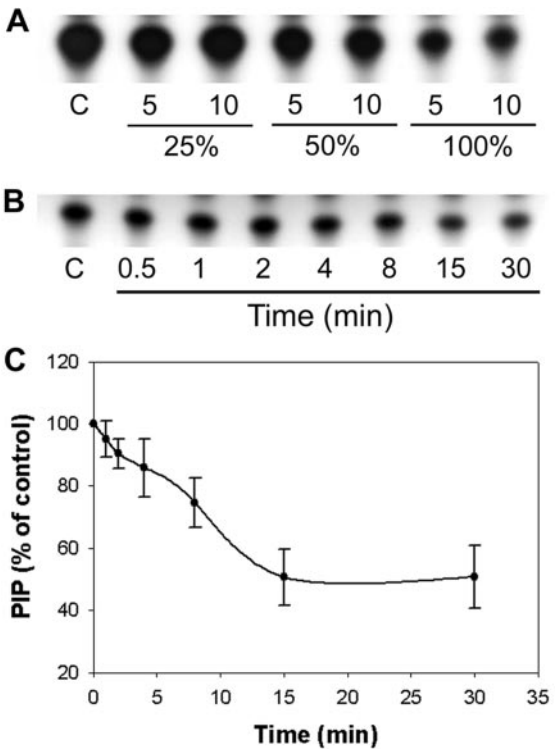

Figure 7. Hypo-osmotic stress induces a decrease in PIP. Lipids were extracted and analyzed by TLC using an alkaline solvent system. A, Concentration-dependent decrease in PIP. Lane 1, Control. Lanes 2 and 3, 5 and 10 min after the addition of 25% (v/v) water. Lanes 4 and 5, 5 and 10 min after the addition of 50% (v/v) water. Lanes 6 and 7, 5 and 10 min after the addition of 100% (v/v) water. B, Time-dependent decrease in PIP. Lane 1, Control. Lanes 2 to 8, 30 s, 1 min, 2 min, 4 min, 8 min, 15 min, and 30 min after the addition of 100% (v/v) water. C, Time course of PIP decrease after addition of 100% (v/v) water.

al., 1999; data not shown). These results indicate that salt-induced hyperosmotic stress stimulates increases in the levels of $PI(3,5)P_2$ and $PI(4,5)P_2$.

Significant changes in the levels of the structural phospholipids PC, PE, and PG could not be detected in response to salt-induced hyperosmotic stress (data not shown).

DISCUSSION

Evidence is emerging that controlled hydrodynamics has an important functional role in the mechanics that drive pollen tube growth (Zonia et al., 2001, 2002; L. Zonia, personal observations). This theory motivated the present investigation to assess whether hypo-osmotic and hyperosmotic shifts in the extracellular medium elicit specific phospholipid signals in growing pollen tubes. The results show that phospholipid signals that are present during normal pollen tube growth are specifically stimulated or reduced as an early response to osmotic perturbation and cell volume changes.

A Number of Phospholipid Signals Are Present during Pollen Tube Growth

Pollen tubes rapidly incorporate $^{32}P_i$ into both signaling and structural phospholipids (Fig. 2). The relative rates of $^{32}P_i$ incorporation are highest for $PI(3,5)P_2$, $PI(4,5)P_2$, PIP, PA, and PI, reflecting a high rate of turnover for phospholipids involved in signaling pathways (Fig. 3). Notably, PA is present during pollen tube growth (Figs. 2 and 3), and it appears to be generated by a constitutively active PLD (Fig. 4). Recently, $PI(4,5)P_2$-dependent and -independent PLD activities were reported in isolated extracts of tobacco pollen tubes (Potocky et al., 2003). The present work significantly demonstrates that PA accumulates to high levels during normal pollen tube growth, has a rapid turnover rate, and is primarily generated through the PLD pathway (Figs. 2–4). In summary, these results show that $^{32}P_i$ labeling enables the detection of three classes of phospholipid signals that are synthesized during pollen tube growth: PIP isomers, PIP_2 isomers, and PA (Figs. 2–4). These phospholipid signals are differentially stimulated or attenuated during responses to osmotic perturbation and cell volume changes. This suggests

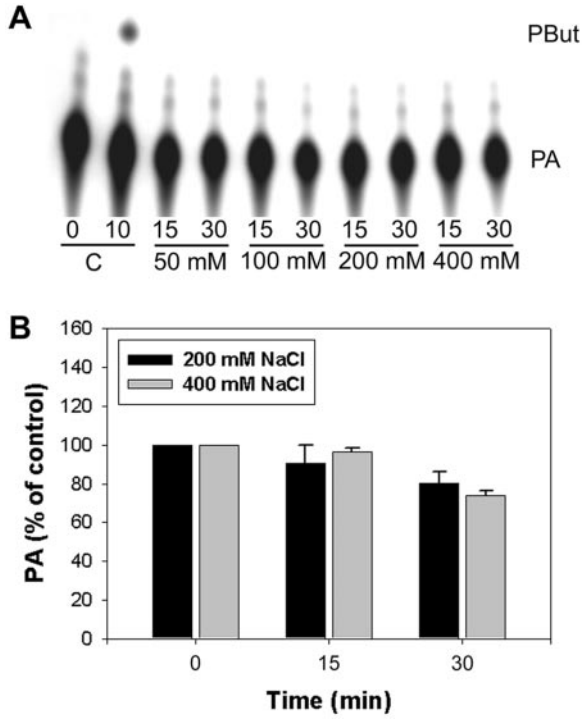

Figure 8. Hyperosmotic stress inhibits PLD activity and reduces the level of PA. Lipids were extracted and analyzed by TLC using an ethyl acetate solvent system. Lanes 1 and 2, Control 0 and 10 min after the addition of 0.1% (v/v) *n*-butanol. Lanes 3 and 4, 15 and 30 min after the addition of 50 mM NaCl. Lanes 5 and 6, 15 and 30 min after the addition of 100 mM NaCl. Lanes 7 and 8, 15 and 30 min after the addition of 200 mM NaCl. Lanes 9 and 10, 15 and 30 min after the addition of 400 mM NaCl. All hyperosmotic stress tests (lanes 3–10) performed in the presence of 0.1% (v/v) *n*-butanol. B, Time-dependent decrease in PA at 200 and 400 mM NaCl.

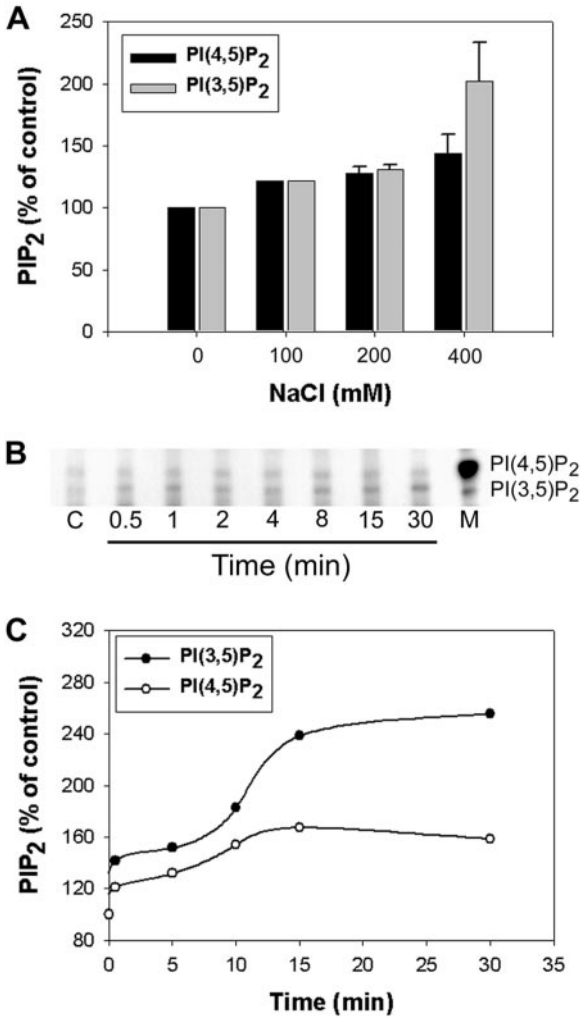

Figure 9. Hyperosmotic stress induces increases in $PI(3,5)P_2$ and $PI(4,5)P_2$. A, Concentration-dependent increases in $PI(3,5)P_2$ and $PI(4,5)P_2$ at 30 min after the start of treatment. Controls, Untreated pollen tubes. B, Time-dependent increases in $PI(3,5)P_2$ and $PI(4,5)P_2$ after addition of 400 mM NaCl. Lipids were extracted and analyzed by TLC using an alkaline solvent system. Lane 1, Control, untreated pollen tubes. Lanes 2 to 8, 30 s, 1 min, 2 min, 4 min, 8 min, 15 min, and 30 min after addition of 400 mM NaCl. Lane 9, Phospholipid marker standard. C, Analysis of time-dependent increases in $PI(3,5)P_2$ and $PI(4,5)P_2$ induced by 400 mM NaCl. Controls, untreated pollen tubes.

that these signals, and perhaps also the mechanisms controlling osmotic status and cell volume, are normal constituents of the networks that drive pollen tube growth.

Three PIP isomers have been identified in plant cells: PI3P, PI4P, and PI5P (Brearley and Hanke, 1992, 1993; Munnik et al., 1994, 1994b; Meijer et al., 1999, 2001b; Pical et al., 1999; DeWald et al., 2001). In addition to acting as substrates/precursors for the synthesis of PIP_2 isomers, PIP species can function as cellular signals involved in membrane trafficking, vacuolar sorting, and cytoskeletal organization (for review, Stevenson et al., 2000; Meijer and Munnik, 2003). Although the structures of the PIP isomers in pollen tubes have not yet been identified, there is evidence for the presence of PI3P (L. Zonia and T. Munnik, unpublished data). The observation of $PI(4,5)P_2$ and $PI(3,5)P_2$ further suggests the presence of PI4P and PI3P. Future work will characterize PIP isomers in pollen tubes.

Three PIP_2 isomers have been reported in plant cells: $PI(3,5)P_2$, $PI(4,5)P_2$, and $PI(3,4)P_2$ (Brearley and Hanke, 1992, 1993; Meijer et al., 1999; Pical et al., 1999; DeWald et al., 2001; Heilmann et al., 2001). The 3,5- and 4,5-phosphorylated isomers have been identified in a number of plant tissues and species, whereas the 3,4-phosphorylated isomer has been identified only in the aquatic plant *Spirodela polyrhiza* (for review, see Meijer and Munnik, 2003 and refs. therein). PIP_2 signals are involved in vesicle trafficking, actin cytoskeleton organization, and vacuolar function (Stevenson et al., 2000; Mueller-Roeber and Pical, 2002; Meijer and Munnik, 2003). In addition, $PI(4,5)P_2$ can be hydrolyzed by PLC, generating the second messengers $Ins(1,4,5)P_3$ and DAG, which in animal cells mobilizes intracellular Ca^{2+} and activates protein kinase C, respectively. In plants, protein kinase C homologs are lacking, and evidence suggests that in several plant tissues, the PLC-generated DAG is rapidly phosphorylated to PA via DAG kinase (Munnik et al., 1998b, 2000; Van der Luit et al., 2000; Den Hartog et al., 2001, 2003; Munnik, 2001; Arisz et al., 2003).

PA has been identified in a number of plant species and tissues, and evidence is accumulating for its role as a phospholipid signal induced by osmotic stress, phytohormones, plant defense elicitors, and wounding (for review, see Munnik, 2001). A main route for the synthesis of PA is through PLD activity (Munnik, 2001; Wang, 2002). PA signaling is involved in protein and lipid kinase activation and ion channel regulation (Munnik et al., 1998a; Farmer and Choi, 1999; Jacob et al., 1999; Lee et al., 2001).

Some Phospholipid Signals Are Specifically Stimulated or Reduced by Hypo-Osmotic Stress and Cell Volume Increase

Pollen tube cell volume undergoes rapid increases after hypo-osmotic treatment (Fig. 1). The cell volume increase displays a hyperbolic response, indicating that water can flow relatively freely into the pollen tube apical region. The results also may imply that a threshold volume of cell swelling must be attained (possibly dependent on elastic properties of the cell wall) before long-term stabilization mechanisms are activated. The pollen tubes do achieve some normal functioning even when subjected to 100% (v/v) water, in that cytoplasmic streaming is only moderately affected and cytoplasmic organization is essentially normal despite the fact that the cells are swollen (data not shown). Much greater

levels of cell swelling than those observed at 100% (v/v) water (a cell volume increase of approximately 45%) are required before pollen tube bursting occurs (a cell volume increase of approximately 58%; Zonia et al., 2002).

Hypo-osmotic stress treatment ≥ 25% (v/v) water rapidly stimulates PLD activity and induces increases in PA (Fig. 5). PA levels increase within 30 s and peak with an average 7-fold increase within 15 to 30 min after treatment with 100% (v/v) water (Fig. 6). Hypo-osmotic stress has previously been shown to induce increased PA levels within 2 min in the unicellular green alga *Dunaliella salina* (Einspahr et al., 1988). Rapid cellular responses to hypo-osmotic stress in plant cells also include changes in Cl^- anion flux across the plasma membrane (Teodoro et al., 1998; Barbier-Brygoo et al., 2000; Shabala et al., 2000). Interestingly, activation of the PLD pathway can potentiate Cl^- secretion in mammalian cells (Vajanaphanich et al., 1993; Oprins et al., 2001, 2002). Low levels of hypo-osmotic treatment induce increases in the frequency and amplitude of Cl^- efflux in tobacco pollen tubes, but with hypo-osmotic stress treatment > 25% to 50% (v/v) water, the magnitude of Cl^- efflux becomes stabilized close to the pretreatment values although the frequency of Cl^- efflux remains high (L. Zonia, personal observation). Taken together, these results may suggest that adjustment to low levels of osmotically induced cell swelling in pollen tubes includes increased Cl^- efflux, whereas adjustment to higher levels of cell swelling includes PLD activation in concert with an increased frequency of Cl^- efflux.

Hypo-osmotic stress ≥ 50% (v/v) water also induces decreases in PIP, with an average 2-fold decrease within 15 to 30 min after treatment with 100% (v/v) water (Fig. 7). Previous work has shown that PIP and PIP_2 levels rapidly decrease after hypo-osmotic stress in *Dunaliella salina* (Einspahr et al., 1988). In pollen tubes, no significant changes in the levels of PIP_2 isomers were observed after hypo-osmotic stress, suggesting that the decrease in PIP may be because of inhibition of its synthesis or because of specific hydrolysis. If PIP could be hydrolyzed by PLC, this could generate $InsP_2$ that could enter inositol polyphosphate metabolic pathways. In addition, altered PIP metabolism could alter the normal distribution of $PI(3,5)P_2$ or $PI(4,5)P_2$, possibly leading to a reorganization of vesicle trafficking or cytoskeletal arrays in response to cell swelling (Gary et al., 1998; Odorizzi et al., 1998; Kost et al., 1999; Wurmser et al., 1999; Meijer and Munnik, 2003).

Other Phospholipid Signals Are Specifically Stimulated or Reduced by Salt-Induced Hyperosmotic Stress and Cell Volume Decrease

Pollen tube cell volume decreases after salt-induced hyperosmotic treatment (Fig. 1). The cell volume response is a linear decrease with increasing levels of hyperosmotic stress from 100 to 400 mM NaCl (Fig. 1). This suggests that pollen tubes can effectively regulate and normalize the internal hydrostatic pressure and apical cell volume when subjected to low levels of salt-induced hyperosmotic stress (<50 mM NaCl) but not when subjected to more severe salt-induced hyperosmotic stress. However, even during the severe plasmolysis that occurs at 400 mM NaCl, a slow rate of cytoplasmic streaming still can be observed in most pollen tubes and indicates that the cells are still viable.

Salt-induced hyperosmotic stress inhibits PLD activity and attenuates PA signaling at all concentrations tested from 50 to 400 mM NaCl (Fig. 8). PA levels decrease by 25% within 30 min after 400 mM NaCl (Fig. 8), whereas they would normally increase by 32% during 30 min of growth in untreated pollen tubes (Fig. 4). This result underscores the specificity of the response to hypo-osmotic stress and cell volume increase, which elicits PLD activation and increases in the levels of PA (Figs. 5 and 6). Although hyperosmotic stress induced by either salt or mannitol in other plant species and tissues has been demonstrated to induce 2- to 3-fold increases in PA (Frank et al., 2000; Munnik et al., 2000; Katagiri et al., 2001; Meijer et al., 2001a, 2002; Munnik, 2001; Munnik and Meijer, 2001; Arisz et al., 2003), a considerable amount of that PA appears to be synthesized through the PLC-DAG kinase pathway (Munnik et al., 2000; Munnik, 2001; Meijer et al., 2002; Arisz et al., 2003). It may be possible that in pollen tubes, the PLC-DAG kinase pathway does not have an important role in the synthesis of PA, even during conditions of hyperosmotic stress. In addition, hyperosmotic stress was shown previously to induce decreased levels of PA in the green alga *Dunaliella salina* (Einspahr et al., 1988).

Salt-induced hyperosmotic stress elicited PIP_2 signals in tobacco pollen tubes, with an average 2-fold increase in $PI(3,5)P_2$ and an average 1.4-fold increase in $PI(4,5)P_2$ within 15 to 30 min after treatment with 400 mM NaCl (Fig. 9). Previous studies in algae and other plant species and tissues have reported increases in either 3,5- and/or 4,5-phosphorylated PIP_2 isomers after hyperosmotic stress (Einspahr et al., 1988; Meijer et al., 1999; Heilmann et al., 1999, 2001; Pical et al., 1999; DeWald et al., 2001). Although the present report does not directly compare the osmotic versus ionic effects of the hyperosmotic treatments, it is significant that previous reports of phospholipid signaling in response to NaCl-induced hyperosmotic stress in other plant cell systems have shown that the responses are because of hyperosmotic stress and not sodium toxicity (Pical et al., 1999; Munnik et al., 2000; Meijer et al., 2001a, 2001b, 2002; Munnik and Meijer, 2001). $PI(3,5)P_2$ is important for vacuolar integrity, tonoplast turnover, and membrane trafficking in yeast (Yamamoto et al., 1995; Gary et al., 1998; Odor-

izzi et al., 1998; Wurmser et al., 1999) and may promote vacuolar reorganization in plants as water moves from the vacuole into the cytosol during hyperosmotically evoked cell volume decrease (Meijer et al., 1999; Munnik and Meijer, 2001; Meijer and Munnik, 2003). PI(4,5)P_2 can associate with the small G-protein Rac and influence vesicle trafficking and growth in pollen tubes (Kost et al., 1999). PLC hydrolysis of PI(4,5)P_2 is blocked when actin-binding proteins such as profilin associate with PI(4,5)P_2 (Machesky and Pollard, 1993; Drøbak et al., 1994; Staiger et al., 1997). However, upon dissociation of the PI(4,5)P_2-profilin complex, reorganization of actin arrays is promoted, and PI(4,5)P_2 is hydrolyzed to generate Ins(1,4,5)P_3 and DAG. Ins(1,4,5)P_3 levels have been shown to increase in plant cells after hyperosmotic stress (Drøbak and Watkins, 2000; DeWald et al., 2001; Takahashi et al., 2001). A potential fate for hyperosmotically induced Ins(1,4,5)P_3 may also be further metabolism to inositol 3,4,5,6-tetrakisphosphate, which inhibits Cl^- efflux and can promote cell volume increase (Nilius et al., 1998; Carew et al., 2000; Ho et al., 2000, 2001; Zonia et al., 2002).

CONCLUSION

This report has demonstrated that pollen tubes respond to extracellular osmotic shifts and osmotically induced cell volume changes by the induction or attenuation of specific phospholipid signals that are present during normal growth. Hypo-osmotic stress and cell volume increase induce increases in PA and reduce PIP. In contrast, salt-induced hyperosmotic stress and cell volume decrease induce increases in PI(3,5)P_2 and PI(4,5)P_2 and reduce PA. Future work will be required to identify the cellular targets for these phospholipid signals and to understand the mechanisms by which they function.

MATERIALS AND METHODS

Pollen Culture and Osmotic Stress Treatments

Pollen from tobacco (*Nicotiana tabacum*) was used for these studies. Anthers were harvested immediately before dehiscence and placed in desiccation chambers for 8 to 12 h. Pollen was collected and stored at −20°C. Pollen was germinated in plastic petri dishes at 23°C on a platform shaker at 50 rpm with a culture density of 1 mg mL^{-1} in germination medium (6% [w/v] Suc, 1.6 mM H_3BO_3, 200 μM $CaCl_2$, and 25 μM MES [pH 5.5]). The osmolarity of germination medium is 0.36 Osm. After labeling the pollen tubes with $^{32}P_i$ (for details, see below), aliquots of the labeled cultures were removed for hypo-osmotic and hyperosmotic studies. Control studies of labeled but untreated pollen tube cultures showed that the normal phospholipid profiles were not affected by transfer of the culture aliquots for experimental treatments. Hypo-osmotic stress was induced by the addition of water to the labeled cultures, so that a 100% (v/v) water stress treatment is a 1:1 (v/v) dilution of the pollen tube culture. Hypo-osmotic stress resulted in the following changes in germination medium osmolarity: 5% (v/v), 0.34 Osm; 25% (v/v), 0.29 Osm; 50% (v/v), 0.24 Osm; and 100% (v/v), 0.18 Osm. Hyperosmotic stress was induced by the addition of the appropriate quantity of a 2.5 M NaCl stock solution. Hyperosmotic stress resulted in the following changes in germination medium osmolarity: 50 mM, 0.46 Osm; 100 mM, 0.56 Osm; 200 mM, 0.76 Osm; and 400 mM, 1.16 Osm.

Cell Volume Measurements

Pollen tube width and apical cell volume were measured as described previously in detail (Zonia et al., 2002). Pollen was germinated essentially as described above except that the density was 0.4 mg mL^{-1} and the cultures were set up in glass petri dishes. Pollen was allowed to germinate and grow for 3.5 to 4.5 h before starting the experiments. The reagent of interest (water or NaCl) was added to the cultures, mixed by gentle swirling, and images of the pollen tube apical regions were captured starting at 5 min after the addition. The cell volume changes rapidly after perturbation of the extracellular osmotic potential, with most of the changes occurring within 5 min. Imaging was performed on a Nikon Eclipse 600 upright microscope with a 40 × 0.6 PlanApo extra-long working distance objective (Nikon, Tokyo) and a high-performance CCD camera (Cohu, Poway, CA). Images were captured using Lucia Image Analysis version 4.7 (Laboratory Imaging, Prague, Czech Republic); 50 images for each experiment were captured within 25 min after the start of treatment. Images were analyzed using Lucia calibrated measurement functions. Each experiment was repeated twice, and the reported values were typical for both experiments. To trace the perimeter of the apical regions of the pollen tubes, a measurement frame was chosen to select the clearly defined boundary of the plasma membrane and a length extending from the apex to 50 ± 0.5 μm distal to the tip. In previous studies, it was established that this apical region of the pollen tube undergoes the most rapid and extensive cell volume changes during osmotic perturbation experiments (Zonia et al., 2002). Area is the principle measurement determined because calibration of the objective provides a direct scaling relationship with the number of pixels selected in the measurement frame. Pollen tube width (W) is then calculated as: $W = A \times L^{-1}$, where A is the area and L is the length. The volume (V) calculation is based on an equivalent right circular cylinder, with radius defined as 0.5 × W and height defined as the length: $V = \pi(0.5 \times W)^2 L$.

Phospholipid Labeling, Extraction, and Analysis

Pollen tube phospholipids were routinely labeled by the addition of carrier-free [^{32}P]orthophosphate ($^{32}PO_4^{3-}$; Amersham International, s'-Hertogenbosch, The Netherlands) at the start of pollen culturing to yield a final concentration of 100 μCi (3.7 MBq) mL^{-1} germination medium. Pollen tubes were allowed to germinate and grow in the presence of $^{32}P_i$ for 4 to 5 h before performing specific osmotic stress experiments with the treatments (for details, see above) and times indicated. After treatment of each experimental sample, 200 μL of pollen tube culture was removed to a 2-mL Eppendorf tube, stopped with a final concentration of 5% (v/v) perchloric acid, and immediately frozen in liquid N_2. Samples were thawed, centrifuged at 9,000*g* for 2 min, and the supernatant was removed. Lipids were extracted by the addition of 400 μL of 50:100:1 (v/v) $CHCl_3$:MeOH:HCl and rigorous vortexing for 1 min before freezing in liquid N_2. Samples were thawed, vortexed for 1 min, and centrifuged as before. The lipid extract was removed to a clean tube and a two-phase system was induced by the addition of 400 μL of $CHCl_3$ and 214 μL of 0.9% (w/v) NaCl. Samples were vortexed for 30 s and centrifuged. The organic phase was removed to a clean tube and washed with an equal volume of 3:48:47 (v/v) $CHCl_3$:MeOH:1 N HCl. Lipid extracts were dried by vacuum centrifugation, resuspended in 20 μL of $CHCl_3$, and stored under N_2 at −20°C.

Phospholipids were analyzed by TLC on Silica 60 TLC plates (Merck, Darmstadt, Germany) using two different solvent systems as described previously (Munnik et al., 1994, 1998b). An alkaline solvent system composed of 45:35:2:8 (v/v) $CHCl_3$:MeOH:25% (v/v) NH_4OH:water was used for chromatography of all phospholipids, whereas an ethyl acetate system, composed of the organic phase of 13:2:3:10 (v/v) ethyl acetate:iso-octane:formic acid:water, was used for chromatography of PA and PBut. Radiolabeled phospholipid marker standards were isolated from hyperosmotically stimulated *Chlamydomonas moewusii* as described previously (Munnik et al., 1994, 2000; Meijer et al., 1999, 2001a). Labeled phospholipids were visualized by autoradiography (X-Omat S, Eastman-Kodak, Rochester, NY) and quantified by phosphor imaging (Storm, Molecular Dynamics, Sunnyvale, CA). Further analysis of the quantified phospholipids was performed using ImageQuant software. The total concentration of ^{32}P for each sample on the TLC plate was calculated, and then this value was normalized with respect to the control values for each experimental sample on the TLC plate. This normalization step allows direct comparison of changes in phospholipids after perturbation studies.

Reagents

Reagents for pollen culturing were plant cell culture or reagent grade and were from Sigma (St. Louis) or Boehringer Mannheim/Roche (Basel). Reagents for lipid extraction and chromatography solvents were from Merck.

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