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Phosphatidylinositol kinases as regulators of GA-stimulated α-amylase secretion in barley (*Hordeum vulgare*)

Ana Laura Villasuso†, Graciela E. Racagni and Estela E. Machado*

Química Biológica, FCEFQN, Universidad Nacional de Río Cuarto, X5804BYA Río Cuarto, Córdoba, Argentina

Correspondence
*Corresponding author,
e-mail: emachado@exa.unrc.edu.ar

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Phosphorylated derivatives of phosphatidylinositol, in association with phosphatidylinositol 3-kinase (PI3 kinase, EC 2.7.1.137) and phosphatidylinositol 4-kinase (PI4 kinase, EC 2.7.1.67), play a key role in regulation of fundamental cell processes. We present evidence for a relationship between α-amylase (EC 3.2.1.1) secretion regulated by GA and levels of phosphatidylinositol 3-phosphate and phosphatidylinositol 4-phosphate (PtdIns(4)P) in barley (*Hordeum vulgare*). Microsomal membranes were incubated in the presence of [γ-^{32}P]ATP, and radiolabeled membrane lipids were extracted and separated by TLC using a boric acid system. Treatment of aleurone layers with GA for short or long periods of time increased PI4 kinase activity. To evaluate the effect of PtdIns(4)P levels on GA signaling, we used phenylarsine oxide (PAO), an inhibitor of PI4 kinase activity. PAO reversibly reduced the α-amylase secretion and protoplast cell vacuolation in a dose-dependent manner. Wortmannin showed a similar inhibitory effect on α-amylase secretion and PI4 kinase activity. GA evoked only a long-term increase in PI3 kinase activity, which was also affected by PAO. The effect of PAO was suppressed by the reducing agent 2,3-dimercapto-1-propanol (BAL), leading to restoration of secretion, vacuolation and PI4 kinase activity. In contrast, the effect of PAO on PI3 kinase activity was not abolished by BAL, suggesting that PI3 kinase is not involved in the secretion process. Likewise, the compound LY294002 inhibited PI3 kinase but had no effect on the secretion process. These findings indicate that PI4 kinase acts as a positive regulator of early GA signaling in aleurone.

Introduction

Highly differentiated aleurone cells synthesize and secrete a variety of hydrolytic enzymes in response to GA during the germination process. To support synthesis of hydrolases before and during secretion, aleurone cells undergo dramatic changes in subcellular organization, such as proliferation of elements of the secretory apparatus, vacuolar coalescence and enlargement and a drop in vacuolar pH that allows mobilized vacuolar storage proteins to fuel hydrolase synthesis (Bethke et al. 1997, Swanson and Jones 1996, Swanson et al. 1998).

Phosphatidylinositol kinases (PI kinases) catalyze the transfer of a phosphate group from ATP to specific positions on the inositol ring of phosphatidylinositol

Abbreviations – BAL, 2,3-dimercapto-1-propanol; PAO, phenylarsine oxide; PI kinases, phosphatidylinositol kinases; PI3 kinase, phosphatidylinositol 3-kinase; PI4 kinase, phosphatidylinositol 4-kinase; PLC, phospholipase C; PSV, protein storage vacuole; PtdInsP, phosphatidylinositol phosphate; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(5)P, phosphatidylinositol 5-phosphate.

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(PtdIns). The PI kinases include phosphatidylinositol 3-kinase (PI3 kinase) and phosphatidylinositol 4-kinase (PI4 kinase), which synthesize phosphatidylinositol 3-phosphate (PtdIns(3)P) and phosphatidylinositol 4-phosphate (PtdIns(4)P), respectively. Plants possess only class III PI3 kinase, which is a homologue of yeast Vps34p. PtdIns(3)P has been implicated in vacuolar trafficking, cell proliferation and cytoskeleton organization (Bunney et al. 2000, Hong and Verma 1994, Welters et al. 1994). PtdIns(3)P levels in plants are normally low but increase rapidly in response to various stimuli (Meijer et al. 1999, Munnik et al. 1994a, 1994b).

PtdIns(4)P formation catalyzed by PI4 kinase is the first step in phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) biosynthesis. Regulation of PI4 kinase activity could therefore affect biosynthesis of not only PtdIns(4)P but also PtdIns(4,5)P_2. In plants, PI4 kinase activity has been purified from several species and found to be present in several different cellular compartments: plasma membrane (Cho et al. 1995, Gross et al. 1992, Sommarin and Sandelius 1988), cytosol (Okpodu et al. 1995), cytoskeleton (Tan and Boss 1992, Xu et al. 1992) and nucleus (Bunney et al. 2000, Hendrix et al. 1989). Two gene types have been cloned from *Arabidopsis thaliana*. The PI4 kinase α variant contains a PH domain able to bind phosphatidic acid, PtdIns(4)P and PtdIns(4,5)P_2; such domain is absent from PI4 kinase β variant (Stevenson et al. 1998, Xue et al. 1999).

Accumulation of phosphatidylinositol 5-phosphate (PtdIns(5)P) induced by osmotic stress has been shown (Meijer et al. 2001), although the enzyme responsible for its synthesis in vivo is not yet known (Mueller-Roeber and Pical, 2002, Sbrissa et al. 2000). A novel Sec14-like protein, PATL1, involved in plant cytokinesis, has been shown to bind phosphoinositides, with a preference for PtdIns(5)P, PtdIns(4,5)P_2 and PtdIns(3)P (Peterman et al. 2004). PtdIns(5)P negatively affects activity of *ATX1* (*Arabidopsis* homolog of trithorax), suggesting a regulatory pathway connecting lipid signaling with nuclear functions (Alvarez-Venegas et al. 2006).

Although PtdIns(3)P, PtdIns(4)P and PtdIns(4,5)P_2 have been shown to be normal constituents of barley aleurone (Brearley and Hanke 1994, Murthy et al. 1989, 1992, Villasuso et al. 2003), their involvement in the secretion process is still poorly understood. Several reports have shown phenylarsine oxide (PAO) to be a specific inhibitor of PI4 kinases, capable of inhibiting the secretion process in mammalian cells (Rajebhosale et al. 2003, Wiedemann et al. 1998). PAO reacts with two thiol groups of closely spaced cysteinyl residues to form stable dithioarsine rings. Interaction between PAO and vicinal dithiol-containing proteins is not affected by monothiols, but in the presence of low-molecular-weight dithiols such as 2,3-dimercapto-1-propanol (BAL) or DTT, the binding is competitively inhibited (Bennett et al. 2000, Sokolovski and Blatt 2004). PAO has been used as a protein tyrosine phosphatase inhibitor in plants (Knetsch et al. 1996, MacRobbie 2002, Olivari et al. 2000, Reyes et al. 2006), but little is known about its function as a PI4 kinase inhibitor in secretion processes.

We previously suggested that early generation of polyphosphoinositides, in association with changes in phosphatidylinositol-specific phospholipase C activity, might be the first step triggering the secretion process, and showed that phosphatidylinositol phosphate (PtdInsP) turnover increases rapidly after GA stimulation (Villasuso and Machado-Domenech, 2004). In this study, we investigated the effect of GA on PtdIns(4)P formation, as related to α-amylase secretion in aleurone cells. We found that PAO affects PI4 kinase activity and reversibly reduces α-amylase secretion and cell vacuolation. Our results suggest that PI4 kinase functions as a positive modulator of GA signaling in cereal aleurone.

Materials and methods

Plant material and amylase assay

Barley grains (*Hordeum vulgare* L cv. Himalaya) were deembryonated, surface sterilized and allowed to imbibe in sterile water for 4 days in the dark at room temperature. Aleurone layers were isolated by gently scraping away the starchy endosperm with a metal spatula. Layers were incubated at 25°C in the presence of 20 m*M* $CaCl_2$ with or without 5 μ*M* GA for 24 h, and α-amylase activity in the incubation medium was measured using the starch-iodine method (Jones and Varner 1967). Amylase activity was determined in the presence of PAO or British Antilewisite (BAL), with addition of DTT, adenosine, wortmannin and LY294002 from 1000-fold concentrated stock solutions. PAO, wortmannin and LY294002 were prepared in DMSO (final DMSO concentration in all cases 0.002% v/v). Agonists were from Sigma (St. Louis, MO). Seeds (cv. Himalaya) were generously provided by Dr R. Zentella (Department of Biology, Washington University, St Louis, MO).

Preparation of crude membrane fractions

Aleurone layers were frozen with liquid nitrogen and homogenized in 10 volumes of buffer A (15 layers in 3 ml of 50 m*M* HEPES, pH 7.4; 0.25 *M* sucrose; 5 m*M* KCl; 1 m*M* EDTA and protease inhibitors: 1 μg ml^{-1} leupeptin; 1 m*M* Phenylmethyl sulfonyl fluoride (PMSF); 1 μg ml^{-1} aprotinin). The homogenate was centrifuged at 1000 *g* for 30 min at 4°C. The supernatant was further centrifuged at

105 000 *g* for 60 min at 4°C. The supernatant was eliminated and the pellet resuspended in 50 m*M* HEPES and used as a crude membrane fraction. Protein concentration of samples was measured with Bradford reagent using BSA as standard (Bradford 1976). Yeast membrane fraction was obtained as described by Stack et al. (1993). Briefly, yeast spheroplasts were resuspended in lysis buffer (0.1 *M* KCl; 15 m*M* HEPES, pH 7.5; 3 m*M* EGTA; 10% glycerol) and then vortexed in the presence of protease inhibitors. The lysate was centrifuged at 750 *g* for 5 min, and the resulting supernatant was centrifuged at 100 000 *g* for 30 min at 4°C to generate Ys (supernatant) and Yp (pellet) fractions. The pellet was resuspended in a volume of lysis buffer equal to that of supernatant, and Ys and Yp were frozen at −80°C until use.

Lipid kinase assay

The membrane fraction isolated as described above (60 μg protein) was added to thermally (30°C) equilibrated 50 m*M* HEPES buffer, pH 7.4; 0.1 m*M* EDTA; 0.5 m*M* DTE; 10 m*M* $MgCl_2$; 0.1 m*M* sodium orthovanadate; 1 m*M* Mg^{2+}-ATP and 370 MBq [γ-^{32}P]ATP. The lipid kinases were assayed simultaneously using endogenous lipids as substrates, unless otherwise stated. Lipid phosphorylation was allowed to proceed for 4 min at 30°C in a final volume of 100 μl. The phosphorylation of endogenous PtdIns was linear for 5 min with 60 μg protein for stimulated and non-stimulated aleurones. Reaction was stopped by addition of 1.5 ml $CHCl_3$/CH_3OH (1:2, v/v) (Racagni-Di Palma et al. 2002). PI3 kinase assay was performed using *Saccharomyces cerevisiae* membrane fraction (approximately 4 μg protein) as control and sonicated PtdIns as substrate (0.2 mg ml^{-1}). Phosphorylated lipids were extracted as described by Stack et al. (1993) and separated in a boric acid system (Walsh et al. 1991). Positions of radiolabeled lipids were determined by autoradiography on Kodak film. Spots were scraped off the plate and counted in a scintillation counter.

SDS–PAGE and Western blot analysis

Secreted amylase was detected by loading aleurone layer incubation medium on gel, while total protein was analyzed from extracts of aleurone tissue. Aleurone layer homogenate was obtained as described above for lipid kinase assay. Medium (M) and layer extracted (E) were separated by 12.5% SDS–PAGE. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes. Protein blots were blocked with 5% skimmed milk powder in PBS (4.3 m*M* $Na_2HPO_4.7H_2O$, 1.4 m*M* KH_2PO_4, 2.7 m*M* KCl, 137 m*M* NaCl). Antibody against α-amylase (a generous gift of Dr Russell Jones, Department of Plant Biology, University of California—Berkeley) was added at 1:1000 and incubated overnight. Secondary antibody coupled to horseradish peroxidase was used with chromogenic substrates (Hwang et al. 2003).

Isolation and stimulation of aleurone protoplasts

Protoplasts were prepared from aleurone layers isolated from de-embryonated barley grains as described previously (Bush and Jones 1988, Bush et al. 1986). Protoplasts were incubated in Gamborg's B-5 medium (release medium), modified by addition of 10 m*M* $CaCl_2$ (Jacobsen et al. 1985). Where indicated, 5 μ*M* GA, 10 μ*M* PAO or 100 μ*M* BAL was included in the incubation medium.

Results

PAO affects α-amylase secretion

We showed recently that GA modifies early generation of polyphosphoinositides, particularly PtdInsP and phosphatidylinositol biphosphate (Villasuso and Machado-Domenech 2004, Villasuso et al. 2003) before secretion can be measured in aleurone layers. This finding suggested that PI4 kinase activity as well as the PtdIns(4)P level may act as a positive regulator of the secretion process in aleurone. To test this hypothesis, PI4 kinase activity was inhibited by PAO and effect of this inhibition was evaluated for Ca^{2+}-dependent pathways of aleurone GA signaling. One of these pathways, α-amylase secretion, was maximal with 5 μ*M* GA treatment at 24 h (approximately 15 ± 1.2 U ml^{-1}). Simultaneous addition of PAO and GA inhibited α-amylase secretion stimulated by GA, in a dose-dependent manner, at 24 h of incubation (Fig. 1A). The inhibitor range tested was 10 n*M* to 100 μ*M*. Inhibition of >45% was found for 10 μ*M* PAO, and this concentration was used for subsequent experiments. IC_{50} value was calculated as 1×10^{-5} *M*. Maximal effect was considered to be reached with an inhibitor range of 50–100 μ*M* when secretion level was similar to that of control ($CaCl_2$). PAO effect in relation to α-amylase secretion was also evaluated by Western blot assay. Reduction of α-amylase activity by PAO was correlated with decreased secretion of α-amylase protein in the incubation medium (Fig. 1A, insert). To determine whether the PAO effect resulted from α-amylase accumulation within the cell of aleurone layers, we also performed protein blotting of aleurone layer extract. PAO treatment did not result in accumulation of α-amylase (Fig. 1A, insert, 'E'). Thus, the total amount of α-amylase synthesized by aleurone layers treated with GA and PAO is less than that for layers treated with GA alone.

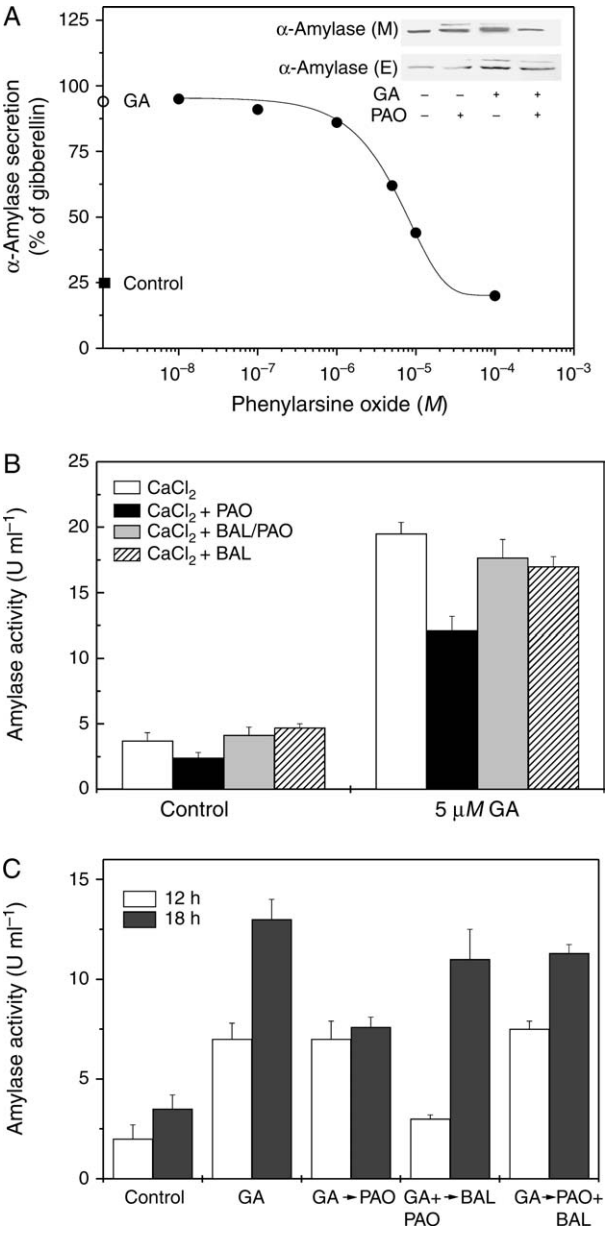

Fig. 1. Effect of PAO on α-amylase secretion. (A) Dose response PAO effect on α-amylase secretion. Aleurone layers were treated with PAO at indicated concentration, plus 5 μ*M* GA (solid circle) or 20 m*M* $CaCl_2$, for 24 h, then α-amylase activity was assayed. $CaCl_2$-treated aleurone layers (solid square) were used as control. Results are expressed as percentage of the maximal α-amylase activity obtained with GA, 16 U ml^{-1} (open circle). Data correspond to one representative experiment of three performed separately. Insert: The amount of α-amylase protein secreted (M) or layer extracted (E) was determined by protein blotting with an antibody against α-amylase. Data shown are from a single experiment representative of at least two replicates. (B) BAL effect on α-amylase secretion. Aleurone layers were treated with 20 m*M* $CaCl_2$ plus 10 μ*M* PAO and/or 100 μ*M* BAL in the presence or absence of GA (control). $CaCl_2$ was added in the incubation with 5 μ*M* GA. α-Amylase activity was assayed at 24 h. Data represent mean ± SD from three independent experiments. (C) Aleurone layers were incubated with 5 μ*M* GA, or 5 μ*M* GA plus 10 μ*M* PAO, for 12 h and then transferred to new medium containing 5 μ*M* GA, 10 μ*M* PAO, 100 μ*M* BAL or 10 μ*M* PAO plus 100 μ*M* BAL, for a 6 h. α-Amylase activity in the incubation medium was measured at 12 and 18 h. Data represent mean ± SD from four independent experiments.

We also assessed the reversibility of PAO effect. PAO can be removed from its targets using small dithiol compounds, causing reversal of its inhibitory effect (Wiedemann et al. 1998). PAO effect was reversed by BAL, that is when 10 μ*M* PAO and 100 μ*M* BAL were added simultaneously to GA-stimulated layers, BAL abolished PAO-induced inhibition of α-amylase secretion (Fig. 1B). BAL alone had no effect on α-amylase secretion.

To determine whether PAO affects particular compartments of the secretory pathway, aleurone layers were incubated with GA or GA plus PAO for 12 h to initiate mobilization of storage proteins necessary for synthesis of α-amylase, which was measured at 12 and 18 h (Hwang et al. 2003). Addition of 100 μ*M* BAL to layers pretreated with GA and 10 μ*M* PAO completely restored the GA effect (Fig. 1C). When PAO was added to layers pretreated with GA, the secretion process was inhibited, suggesting an effect on some compartment(s) of the secretory pathway. In contrast, the effect of simultaneous addition of 10 μ*M* PAO and 100 μ*M* BAL to layers pretreated with GA was not significantly different from that of control (GA treatment alone). These findings indicate that the secretory pathway for exocytosis of α-amylase functions normally when PAO effect is competitively inhibited by BAL.

We also tested release of amino acids as another indicator of storage protein breakdown. In a previous study, total ninhydrin-reactive materials accumulating in the medium surrounding aleurone layers increased almost five-fold in response to GA (Hwang et al. 2003). In layers treated with GA and PAO, we observed approximately 35% decrease in release of amino acids to medium, probably because of inhibited proteolysis of storage proteins.

PAO prevents GA-regulated vacuolation

To support synthesis and secretion of α-amylase, the aleurone cell mobilizes stored proteins and other vacuolar reserves in response to GA (Bethke and Jones 1998). The vacuolation process, that is formation of a large vacuole, is indicative of the GA response (Bethke et al. 1997). Reduction of vacuolation may result from inhibited mobilization of storage proteins of protein storage vacuole (PSV) and reduced amino acid pool size. We evaluated PAO effect on various vacuolation stages, by treating isolated protoplasts with GA in the presence or

absence of 10 μ*M* PAO and 100 μ*M* BAL. Protoplasts with stages I and III vacuole profiles were associated with morphology of non-GA-treated cells and cells with full GA response, respectively (Fig. 2A, B). Typical cytological changes induced by GA, that is coalescence and vacuole enlargement, were not observed in PAO-treated cells (Fig. 2C). Quantification of vacuole size showed that 10 μ*M* PAO treatment resulted in inhibition of vacuole enlargement, while addition of 10 μ*M* PAO plus 100 μ*M* BAL blocked PAO effect on GA-stimulated vacuolation (Fig. 2D).

PAO inhibits phosphoinositide kinase activity during α-amylase secretion

To further explore the role of PI4 kinase in secretory activity of barley aleurone, we assayed the kinase activity when α-amylase secretion was maximal, at 24 h. The kinase activity was assessed in aleurone layers treated with 5 μ*M* GA in the presence or absence of 10 μ*M* PAO. The PtdIns(4)P isomer was identified initially. After the kinase activity was measured, lipids were extracted and separated by TLC-borate, based on the ability of boric acid to form complexes with 2,3-*cis*-diols present in the inositol ring of PtdIns and PtdIns(4)P. Complex formation is inhibited in the 3-isomers because the 3-OH is substituted by phosphate group.

Fig. 3 shows autoradiography of a typical experiment where two spots were found, the upper and lower, respectively, representing putative PtdIns(3)P and PtdIns(4)P. The Rf values, 0.50 and 0.45 for PtdIns(3)P and PtdIns(4)P, respectively, were slightly different from those reported by Walsh et al. (1991). Analysis of radioactive lipids showed PI4 kinase activity (5 pmol mg^{-1} protein min^{-1}) approximately 2.5-fold higher than PI3 kinase activity (2 pmol mg^{-1} protein min^{-1}) in aleurones without

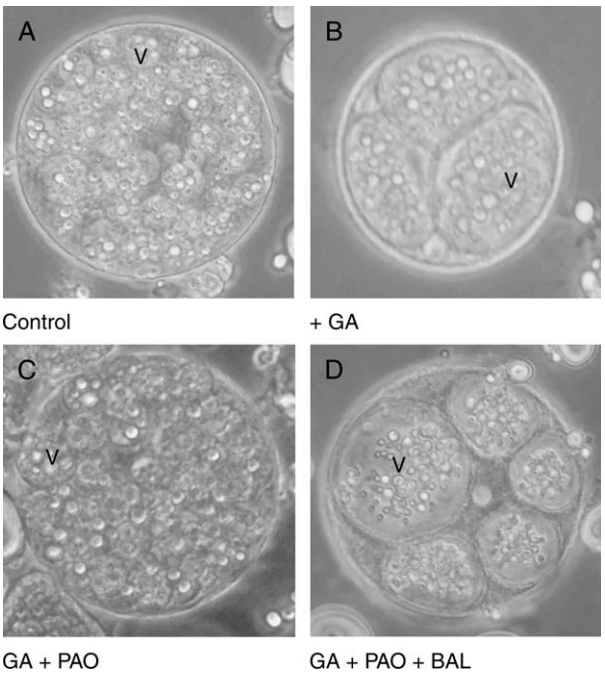

Fig. 2. Effects of PAO and BAL on GA-stimulated vacuolation of protoplasts. Phase contrast microscopic images of aleurone protoplasts treated with (A) 20 m*M* $CaCl_2$ (control), stage I; (B) 5 μ*M* GA, stage III; (C) 5 μ*M* GA plus 10 μ*M* PAO; (D) 5 μ*M* GA plus 10 μ*M* PAO and 100 μ*M* BAL. All incubations were carried out for 24 h in the presence of 20 m*M* $CaCl_2$.

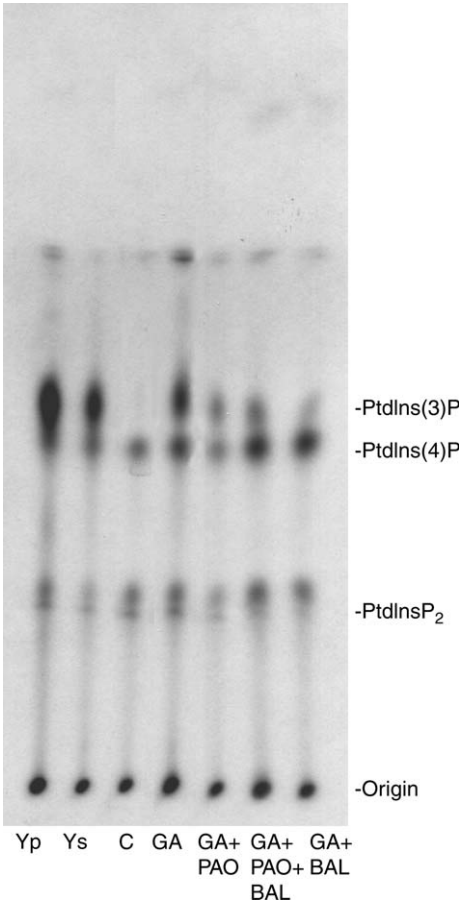

Fig. 3. Effects of PAO and BAL on activities of PI3 and PI4 kinases in barley aleurone. Aleurone layers were treated with 20 m*M* $CaCl_2$, 5 μ*M* GA, 5 μ*M* GA plus 10 μ*M* PAO, 5 μ*M* GA plus 10 μ*M* PAO and 100 μ*M* BAL or 5 μ*M* GA plus 100 μ*M* BAL for 24 h. Membrane microsomes (60 μg protein) were incubated in the presence of [γ-^{32}P]ATP as described in Materials and methods. Membrane lipids were extracted and separated by TLC using a boric acid system and visualized by autoradiography. Proteins prepared from yeast [4 μg of yeast soluble (Ys)] and membrane homogenate (Yp) were assayed for kinase activity to generate reference markers for PtdIns(3)P and PtdIns(4)P. C indicates 20 m*M* $CaCl_2$-treated aleurone (control).

stimulation. This suggests that PtdIns(3)P level in aleurone is lower than PtdIns(4)P level, consistent with findings in other cell systems (Meijer et al. 2001, Munnik et al. 1994a, 1994b). However, GA increased PI4 kinase activity approximately five-fold relative to control (22–24 vs 4–5 pmol mg^{-1} protein min^{-1}) after 24 h of GA treatment, indicating that the kinase activity is modulated by the hormone. Tests for short time periods showed that GA at 5 min did not modify PI3 kinase activity but greatly increased PI4 kinase activity (data not shown). Consistent with our previous reports (Villasuso and Machado-Domenech 2004), GA increased PI4 kinase activity approximately six-fold, a value similar to that reached at 24 h.

In the presence of PAO, PI4 kinase activity fell to 10 pmol mg^{-1} protein min^{-1}, a 60% reduction. We determined that PtdIns(4)P levels, as a consequence of PI4 kinase activity higher than 10 pmol mg^{-1} protein min^{-1}, would be necessary for full secretory activity typical of a GA signal response. BAL restored the kinase activity and increased the specific activity. BAL incubation by itself had no effect on α-amylase secretion or PI4 kinase activity.

The boric acid solvent system allowed us to determine that PI3 kinase activity was modified by GA. When this kinase was assayed under the same conditions used for PI4 kinase, it was clear that GA also increased the PI3 kinase activity, which reached the value of PI4 kinase activity after 24 h of GA treatment. When layers were treated with PAO, PI3 kinase activity was also reduced about 50%.

Amylase activity reached maximal value in the presence of BAL and PAO (Fig. 1B), indicating that reduction of PtdIns(3)P levels by PAO did not affect the secretion process. In our studies of BAL effect on PI3 kinase activity during the secretion process, it was clear that dithiol compounds were unable to reverse the inhibitory effect of PAO. These observations suggest that only PI4 kinase activity triggers the α-amylase secretion process while maintaining typical GA response. However, we cannot rule out the possibility that PAO effect on PI3 kinase activity is because of another mechanism.

To determine whether amount of endogenous substrate was a limiting factor for reaction rate in the kinase activity assay, we performed a series of experiments with addition of exogenous PtdIns. When 0.2 mg ml^{-1} PtdIns was added, no change was observed in PtdIns(3)P level, regardless of the presence or absence of GA. However, increased PtdIns(4)P and PtdIns(4,5)P_2 levels were observed, suggesting that formation of PtdIns(4,5)P_2 depends on availability of PtdIns(4)P (data not shown).

Wortmannin and LY294002 affect phosphoinositide kinase activities and the secretion process

We also evaluated the effects of other inhibitors of PI kinase activity. There are two types of PI4 kinases, which differ in their sensitivity to micromolar concentrations of wortmannin and in their Ca^{2+} dependence. No type II PI4 kinase in plants has been biochemically characterized yet (Krinke et al. 2007). According to studies in animals and yeast (*S. cerevisiae*), type II PI4 kinases are inhibited by high Ca^{2+} concentration but not by micromolar concentrations of wortmannin. In contrast, type III PI4 kinases are inhibited by micromolar concentrations of wortmannin but are not affected by Ca^{2+} (Mueller-Roeber and Pical, 2002). PI4 kinase activity and α-amylase secretion were reduced 50% in the presence of 6 μ*M* wortmannin (Fig. 4A), showing again the importance of PI4 kinase during the secretion process.

Adenosine sensitivity has also been used as a tool for characterization of PI4 kinase. In other systems, adenosine has been used for inhibition at concentrations ranging from 20 μ*M* for type II PI4 kinase to 1.5 m*M* for type III PI4 kinase. In this study, PI4 kinase retained 85% of its activity in the presence of 300 μ*M* adenosine (Fig. 4B), suggesting that activity of a type III PI4 kinase may be responsible for the observed PtdIns(4)P decrease.

Plant PI3 kinases are inhibited by submicromolar concentrations of wortmannin and by LY294002 (Turck et al. 2004). Low concentrations of wortmannin (up to 1 μ*M*; Fig. 4C) or 50 μ*M* LY294002 (Fig. 4D) did not affect the response to GA, although they caused approximately 30% or approximately 40% inhibition of PI3 kinase activity, respectively. These findings suggest that PI3 kinase is not responsible for the observed changes.

Discussion

Our present results show a relationship between the PtdIns pathway and the GA-stimulated secretion process. Previous biochemical and pharmacological studies suggested that the inositol cycle, phospholipase C (PLC) activity and associated lipid kinases are important regulators of GA response in the cereal aleurone (Villasuso and Machado-Domenech, 2004, Villasuso et al. 2003). GA causes an increase in cytoplasmic Ca^{2+} and PLC activity, which are required for secretion of α-amylase, although they are not required for α-amylase gene transcription (McCubbin et al. 2004, Villasuso et al. 2003). PtdInsP level in aleurone cells fluctuated considerably during GA treatment, reaching a maximal percentage of control value at 5 min. We hypothesized that regulation of PI4 kinase activity affects biosynthesis

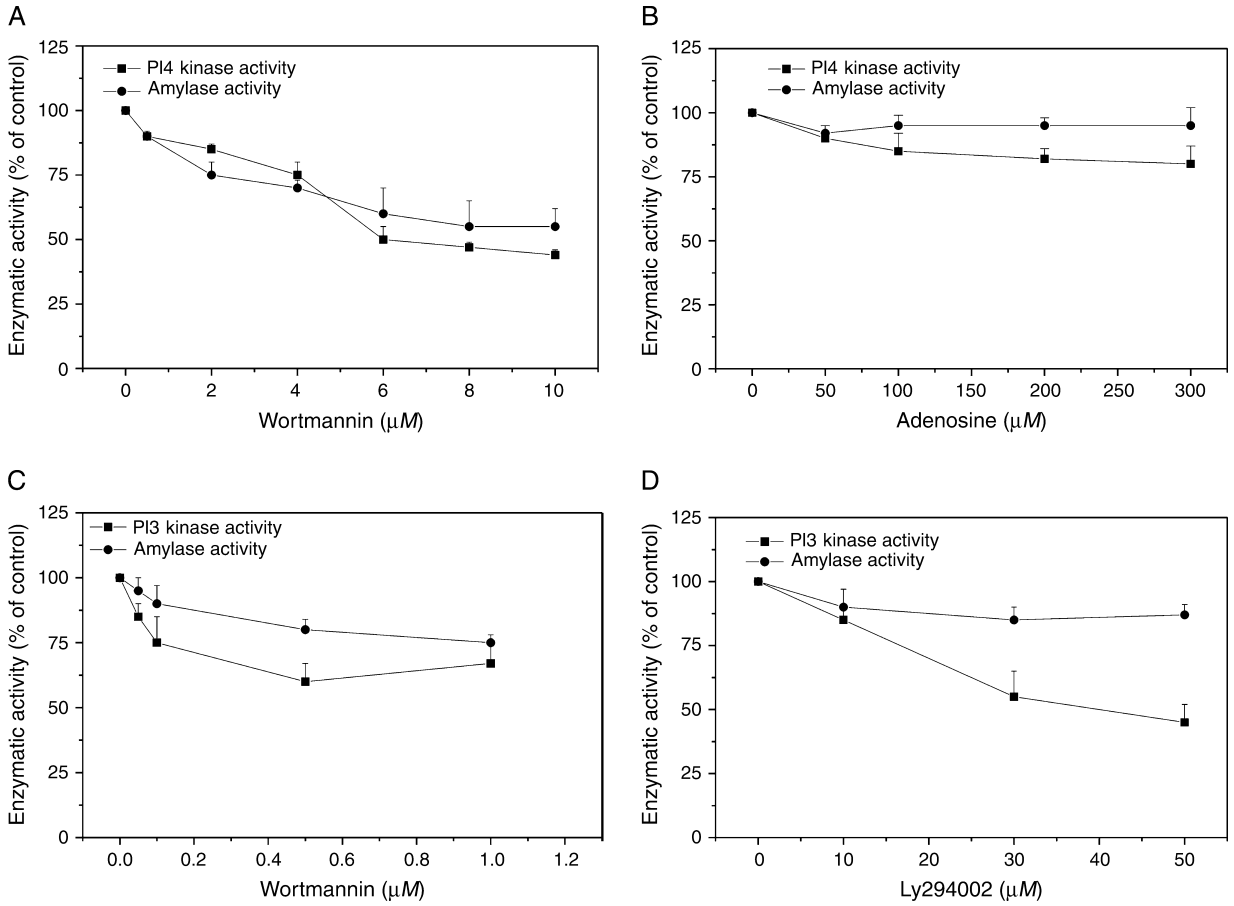

Fig. 4. Wortmannin, adenosine and LY294002 sensitivity of PI3 and PI4 kinases activities in aleurone layers treated with 5 μ*M* GA. Amylase and PI4 kinase activities at 24 h: effect of (A) wortmannin and (B) adenosine. Amylase and PI3 kinase activities at 24 h: effect of (C) wortmannin and (D) LY294002. Data represent mean ± SD from three independent experiments, expressed as percentage of control.

of not only PtdIns(4)P but also PtdIns(4,5)P_2 and consequently the secretory activity of aleurone. We found that α-amylase secretion was affected by PAO and wortmannin in a dose-dependent manner when barley aleurone PI4 kinase activity was inhibited, suggesting that the secretion process in aleurone depends on PtdIns(4)P level (Villasuso and Machado-Domenech 2004, Villasuso et al. 2003).

The ability of BAL to reverse the inhibitory effect of PAO on PI4 kinase activity and α-amylase secretion indicates an interaction between PAO and a vicinal dithiol of PI4 kinase. DTT, another known PAO competitor, also reversed the inhibitory effect of PAO. The secretion process was affected at various levels when PI4 kinase was inhibited by PAO. A key feature of aleurone cell response to GA is the fusion of small PSV to form a large central vacuole (Bethke and Jones 1998, Zentella et al. 2002). We showed that reduction of PtdIns(4)P level also affects the GA-stimulated vacuolation process in aleurone protoplasts. The kinase inactivated by PAO appeared to inhibit GA response, thus shifting the vacuolation process toward stage I. Competitive interaction of the enzyme by BAL overcame that effect and drove the vacuolation process to full GA response, that is stage III.

Formation of a central vacuole is preceded by mobilization of stored protein reserves, and vacuole acidification is necessary for breakdown of those stored proteins because many proteases isolated from PSV have acidic pH optima (Bethke and Jones 1998, HaraNishimura et al. 1998). The effect of PAO on vacuolation may result from changes in PSV proteolytic activity because a decrease in amino acid release was observed. This suggests a link between GA effect on mobilization of stored reserves and phosphoinositide formation, specifically of PtdIns(4)P.

To clarify direct or indirect effects of PAO on the secretory pathway, storage protein breakdown and α-amylase synthesis were initiated by pretreating aleurone layers with GA for 12 h to overcome a possible

limitation of amino acid pool. We expected α-amylase secretion to reach maximal level; however, secretion remained low. These results suggest that PtdIns(4)P levels play an essential role in protein transport to the cell exterior via the endomembrane system. This idea is supported by Western blot results showing that treatment of cells with PAO affected the total level of α-amylase (Fig. 1A, insert). The correlation between decreased GA response, α-amylase secretion and PI4 kinase activity strongly suggests that in the Ca^{2+}-dependent secretory pathway, metabolism of PtdIns(4)P is necessary for triggering secretion.

Sensitivity to adenosine treatment and wortmannin in aleurone suggested the presence of a type III PI4 kinase, which is the prevalent PI4 kinase activity in plants (Krinke et al. 2007, Mueller-Roeber and Pical 2002). PtdIns(4,5)P_2 formation may also depend on PtdIns(4)P level because addition of exogenous PtdIns to kinase assay increased PtdIns(4,5)P_2 level, and PAO treatment decreased its level. Therefore, we cannot rule out the possibility that synthesis and metabolism of PtdIns(4,5)P_2 are involved in regulation of the secretion process.

Although PAO has been widely used as inhibitor of protein tyrosine phosphatases and caspases (Knetsch et al. 1996, MacRobbie 2002, Olivari et al. 2000, Reyes et al. 2006), in this study, we provide evidence that this compound is capable of inhibiting PI4 kinase without excluding a possible PAO effect on others enzymes. Under our experimental conditions, it is unlikely that the caspase activity is affected by PAO because the VEIDase caspase-like proteolytic activity described in barley caryopsis has not been detected in aleurone germination (Borén et al. 2006). By taking account that the ABA antagonist effect may be mediated by a PTPase (Knetsch et al. 1996), we believe that the secretion should be increased in the presence of PAO. However, the secretion was inhibited. Based on the results and in combination with the available literature, we suggest that the effect of PAO on PTPase does not involve in the secretion modulation.

In our studies of factors affecting GA response, PtdIns(3)P level at 5 min was found to be much lower than at 24 h of stimulation. We therefore speculated that as this level increased at 24 h, PtdIns(3)P might become involved in the secretion process. However, we found that reduction of PtdIns(3)P level had no effect on the secretion process. BAL did not reverse the inhibitory effect of PAO. Furthermore, neither LY294002 nor Wortmannin blocked the typical GA response when applied at a concentration capable of inhibiting PI3 kinase activity (Mueller-Roeber and Pical 2002). Thus, a correlation was not found between PtdIns(3)P level and secretory activity of aleurone.

In summary, the present results show that activity of PI4 kinase is necessary for the capability of aleurone to secrete α-amylase during GA stimulation.

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