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RESEARCH PAPER

Phosphoinositides and phosphatidic acid regulate pollen tube growth and reorientation through modulation of $[Ca^{2+}]_c$ and membrane secretion

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Abstract

The maintenance of a calcium gradient and vesicle secretion in the apex of pollen tubes is essential for growth. It is shown here that phosphatidylinositol-4,5-bisphosphate (PIP_2) and D-myo-inositol-1,4,5-trisphosphate (IP_3), together with phosphatidic acid (PA), play a vital role in the regulation of these processes. Changes in the intracellular concentration of both PIP_2 and IP_3 (induced by photolysis of caged-probes), modified growth and caused reorientation of the growth axis. However, measurements of cytosolic free calcium ($[Ca^{2+}]_c$) and apical secretion revealed significant differences between the photorelease of PIP_2 or IP_3. When released in the first 50 μm of the pollen tube, PIP_2 led to transient growth perturbation, $[Ca^{2+}]_c$ increases, and inhibition of apical secretion. By contrast, a concentration of IP_3 which caused a $[Ca^{2+}]_c$ transient of similar magnitude, stimulated apical secretion and caused severe growth perturbation. Furthermore, the $[Ca^{2+}]_c$ transient induced by IP_3 was spatially different causing a pronounced elevation in the sub-apical region. These observations suggest different targets for the two phosphoinositides. One of the targets is suggested to be PA, a product of PIP_2 hydrolysis via phospholipase C (PLC) or phospholipase D (PLD) activity. It was found that antagonists of PA accumulation (e.g. butan-1-ol) and inhibitors of PLC and PLD reversibly halted polarity. Reduction of PA levels caused the dissipation of the $[Ca^{2+}]_c$ gradient and inhibited apical plasma membrane recycling. It was also found to cause abolition of the apical zonation. These data suggest that phosphoinositides and phospholipids regulate tip growth through a multiple pathway system involving regulation of $[Ca^{2+}]_c$ levels, endo/exocytosis, and vesicular trafficking.

Key words: $Ins(1,4,5)P_3$, phosphatidic acid, phospholipases, PIP_2, secretion.

Introduction

Pollen tubes are characterized by extreme polar growth and multiple signalling pathways are required for its maintenance (Malhó *et al.*, 2000; Holdaway-Clarke and Hepler, 2003). Targeted vesicle fusion, Ca^{2+}, protein kinases, cAMP, GTPases, and specific cytoskeleton arrangements have been documented to play crucial roles in apical growth (Moutinho *et al.*, 2001; Vidali and Hepler, 2001; Camacho and Malhó, 2003). Although phosphoinositides are a vast family of molecules playing a major role in signalling, their contribution for polar growth is still largely unknown (Malhó and Camacho, 2004). PIP_2 has been shown to act in a common pathway to Rac GTPases (Kost *et al.*, 1999) and IP_3 is known to modulate Ca^{2+} levels (Franklin-Tong *et al.*, 1996; Malhó, 1998). Inositol 3,4,5,6-tetra*kis*phosphate [$Ins(3,4,5,6)P_4$] was found to disrupt Cl^- efflux (Zonia *et al.*, 2002) and Potocký *et al.* (2003) showed that apical growth depended on PLD activity. Therefore, it seems that

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Abbreviations: $[Ca^{2+}]_c$, cytosolic free calcium; IP_3, D-myo-inositol-1,4,5-trisphosphate; PA, phosphatidic acid; PIP_2, phosphatidylinositol-4,5-*bis*phosphate; PLC, phospholipase C; PLD, phospholipase D.

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phosphoinositides intersect multiple signalling pathways and are key regulators of polarity.

The intact PIP_2 molecule is a central player in actin dynamics, vesicle trafficking, and ion transport (Cremona *et al.*, 1999; Stevenson *et al.*, 2000) due to its ability to bind and regulate many proteins containing PIP_2 recognition domains such as pleckstrin homology domains, basic patches, and epsin N-terminal homology domains (Martin, 1998; Cockcroft and De Matteis, 2001). Through PLC, PtdIns(4,5)-P_2 generates IP_3 and diacylglycerol (DAG) which can be converted to PA through DAG kinase (Munnik, 2001). PIP_2 is also known to govern PLD activity leading to elevated PA formation (Powner and Wakelam, 2002). Multiple PLD genes have been identified in plants and the proteins they code for seem to be regulated by Ca^{2+} and G-proteins (Zheng *et al.*, 2000; Munnik, 2001). Activation of plant PLDs is triggered by various cues, namely pathogen elicitation (Young *et al.*, 1996) and a pollen signalling protein (PsiP) involved in cAMP production sharing great homology with defence proteins was recently described (Moutinho *et al.*, 2001).

IP_3, possibly the most studied signalling phosphoinositide, is a potent mobilizer of Ca^{2+} from intracellular stores (Martin, 1998). In pollen tubes, an IP_3-induced Ca^{2+} release seems to be required for the transduction of signals from the apex to further regions of the cell (Malhó, 1998). It was further suggested that IP_3 receptors may have an asymmetric activity depending on their spatial localization: in the apex, where Ca^{2+} is elevated, the receptor undergoes an intrinsic inactivation when IP_3 is bound; in sub-apical regions, where Ca^{2+} is in the nM range, increasing $[Ca^{2+}]_c$ potentiates Ca^{2+} release by IP_3 to the extent that Ca^{2+} and IP_3 can be regarded as co-agonists for Ca^{2+} release (Dawson, 1997). In animal cells, IP_3 receptor-like proteins were shown to be linked to actin filaments (Fujimoto *et al.*, 1995) linking phosphoinositides to cytoskeleton organization.

PA is an end-product of PIP_2 hydrolysis via PLC or the promotion of PLD activity. This phospholipid promotes membrane curvature and the formation of secretory vesicles together with a crucial role in the structural integrity of the Golgi (Sweeney *et al.*, 2002). It has also been demonstrated that continual production of PA is essential for cytoskeleton reorganization (O'Luanaigh *et al.*, 2002). As part of a feedback loop, PA can promote PIP_2 formation by phosphatidylinositol 4-phosphate 5-kinase (Anderson *et al.*, 1999).

The existence of a putative signalling cascade involving phosphoinositides and their targets in polar growth has been addressed here. Using caged-probes and specific inhibitors, the intracellular levels of PIP_2, IP_3, and PA in growing pollen tubes has been modulated. The data suggest that in these cells both IP_3 and PA are formed as a result of PtdIns(4,5)-P_2 conversion. These three signalling molecules have a concerted action modulating the tip-focused $[Ca^{2+}]_c$ gradient, membrane secretion, and cytoskeleton organization, thus playing a key role in the establishment and maintenance of polarity.

Materials and methods

Plant material

Unless otherwise stated, pollen of *Agapanthus umbellatus* was harvested, stored, and pollen tubes were grown *in vitro* as described previously (Malhó and Trewavas, 1996; Camacho and Malhó, 2003). Pollen tubes were germinated in semi-solid growth medium containing 0.01% H_3BO_3, 0.02% $CaCl_2$, 0.02% KCl, 0.02% $MgCl_2$, 2.5% (73 mM) sucrose, and 0.8% Agarose II (Sigma), pH 6.0.

Loading and localized photolysis of caged PIP_2 and caged IP_3

Caged PIP_2 and caged IP_3 (Calbiochem, Nottingham, UK) were loaded into pollen tubes following the method described by Rato *et al.* (2004). Briefly, pollen grains were submitted to a 900 mM mannitol (Sigma) osmotic shock for 30 min in a medium containing 50–100 μM caged PIP_2 or 20–50 μM caged IP_3. After this period, the cells were transferred to semi-solid growth medium and left to germinate as described previously. Estimates for the intracellular concentration of the caged-probes were performed using caged-fluorescein as described by Malhó and Trewavas (1996). Briefly, the fluorescence emitted after photoactivation of known concentrations of caged-fluorescein was compared with the fluorescence emitted by the fluorescein loaded into pollen tubes (further details can be found in the supplementary information at JXB online).

To photoactivate the caged reagents locally, a 360 nm UV light pulse was focused on an irradiation area of ~80–95 μm^2 diameter (using an iris diaphragm placed in the excitation filter wheel). Pollen tubes were then exposed to 5 s UV pulses in selected regions of the cells.

Bright field imaging

Bright field and/or DIC images were acquired with a PCO Sensicam-QE camera (Labocontrole, Lisbon, Portugal) attached to an Olympus IX-50 microscope (Labocontrole, Lisbon, Portugal) using an Olympus X40 UplanApo (NA=0.85) objective. The interval between image acquisition and the exposure time was controlled through Image Pro Plus 5.0 software (Media Cybernetics, Leiden, The Netherlands).

Modulators of intracellular PA levels

The primary alcohol butan-1-ol (VWR International, Darmstadt, Germany) was dissolved in growth medium to a final concentration of 100 mM. A similar solution was made for butan-2-ol. Aliquots of the PLC inhibitor U73122 (10 mM stock solution, Calbiochem, Mannheim, Germany) dissolved in chloroform (100%) were mixed with growth medium resulting in a final concentration of 250 μM, and stirred for 15–30 min to evaporate the chloroform. The DAG kinase inhibitor R53022 (Calbiochem) was made up as stock solution in dimethylsulphoxide (10 mM). Aliquots were diluted with growth medium to a final concentration of 150 μM. The resulting dimethylsulphoxide concentration (1%) has no effect on pollen tube germination and growth (Malhó *et al.*, 1994). PA (Sigma Aldrich, Munich, Germany) was primarily dissolved in 25 μl dimethylsulphoxide before the addition of growth medium to produce a stock solution of 28.5 mM which was further diluted to 7.2 mM (0.65% dimethylsulphoxide in the growth medium).

Confocal ratio imaging of $[Ca^{2+}]_c$

Ca^{2+}-sensitive fluorescent dye Calcium Green-1 and the Ca^{2+}-insensitive fluorescent dye Rhodamine B, both conjugated with a

10 kDa dextran (1 mM, Molecular Probes, Eugene, UK), were loaded into pollen tubes through pressure microinjection as described previously (Camacho *et al.*, 2000). For some experiments, the microneedles were co-filled with 0.7 mM caged PIP_2 or 0.3 mM caged IP_3. Details of the experimental procedure and criteria used to establish the success of microinjection can be found in Malhó *et al.* (1994). $[Ca^{2+}]_c$ ratio imaging was performed using a Bio-Rad MCR-600 (Microscience Ltd, Hemel Hempstead, U.K) confocal laser scanning microscope (CLSM) operating in the dual channel mode as described in Camacho *et al.* (2000). Ratio images were calculated with the TCSM/MPL software (Bio-Rad Microscience Ltd.) and then quantified in terms of average pixel intensity (0–255 scale for 8 bit images).

FM 1-43 labelling and confocal imaging

Labelling and FM 1-43 confocal imaging was performed as described previously (Camacho and Malhó, 2003; Rato *et al.*, 2004). Briefly, pollen tubes were labelled with 0.2 μM FM 1–43 (Molecular Probes) and thin time-course optical sections (~5 μm thick) acquired with a CLSM. Fluorescence was quantified in terms of average pixel intensity.

Actin labelling

Pollen tubes were fixed with the cross-linking agent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (100 μM, MBS, Sigma Aldrich, Munich, Germany) in 100 mM PIPES buffer (pH 6.8) containing EGTA (10 mM) $MgSO_4$ (5 mM) and Triton-100 (0.05%) at room temperature for 30 min (Sonobe and Shibaoka, 1988). After three washes in PIPES buffer (50 mM, pH 6.8) without Triton-100 the probes were labelled with rhodamin-phalloidin (0.825 nM, Molecular Probes Inc., USA) dissolved in PIPES buffer.

Transmission electron microscopy

Germinated pollen tubes were fixed simultaneously with 2% para-formalehyde and 2% glutaraldehyde in cacodylate buffer (75 mM, pH 7.0) for 90 min, transferred into 2% agar and then post-fixed with 1% osmium tetroxide at 4 °C for 12–14 h. The samples were de-hydrated through a series of graded ethanol concentrations, 7.5–100%, and finally embedded in plastic according to Spurr (1964). Ultrathin sections were cut with a ultramicrotome (Ultracut E, Reichert-Jung, Vienna, Austria) and stained with uranyl acetate/lead citrate. Sections were viewed with a LEO 906 E transmission electron microscope (LEO, Oberkochen, Germany) equipped with a Gatan MultiScan CCD Camera (Munich, Germany). Images were acquired using the Digital Micrograph 3.3 software (Gatan).

Data analysis

Growth rates and fluorescence intensity were measured using Image-Pro Plus 4.0 software. The fluorescence measurements presented correspond to medium fluorescence intensity in the first 0–10 μm and 10–20 μm of the pollen tube apex (apical and sub-apical regions, respectively).

Unless specifically mentioned, numerical data in the figures correspond to single cell analysis of typical experiments and not to summary statistics. This is because there is a significant degree of variability at a biological level, but also at a technical one; even minor changes in the degree of loading, amount of photolysed molecule, area of release, disturbance on microinjection, and responsiveness of the cell can play a role in the extent of cellular response (Malhó and Trewavas, 1996). For measurements on germination rate and growth rates, a one-way analysis of variance (ANOVA, P <0.05) was applied.

Results

Intracellular changes in PIP_2 and IP_3 modify pollen tube growth rate and axis orientation

To study the role of phosphoinositides in the regulation of pollen tube growth, caged versions of PIP_2 and IP_3 were loaded into *Agapanthus* pollen tubes using an osmotic shock treatment (Rato *et al.*, 2004). The caged-probes were subsequently photoreleased in discrete regions of the cell with a 5 s UV pulse (360 nm) focused through an iris diaphragm. Since these probes are not fluorescent, it is impossible to determine their intracellular concentration upon photolysis. Estimates can, however, be provided using caged-fluorescein (Malhó and Trewavas, 1996); this probe becomes fluorescent upon photorelease and the light emitted from loaded cells can be compared with known concentrations of the fluorophore from *in vitro* solutions (for supplementary information see JXB online).

Both phosphoinositides were found to cause reorientation of the growth axis when photorelease was performed at one side of the apical dome (Fig. 1). Controls involved exposing unloaded cells to the same UV pulse (n=5) that revealed no effect (Rato *et al.*, 2004). In cells loaded with ~0.5–0.8 μM of caged PIP_2 (n=32), 43.7% changed their growth direction towards the side of higher PIP_2 (Fig. 1) while the remaining cells showed no effect. An equal concentration of IP_3 caused abrupt decreases of growth rates accompanied by abnormal tip morphology and often tip bursting. This reveals that, as reported for animal cells (Bird *et al.*, 1992), within the same range of concentration, IP_3 is more effective inducing physiological responses than PIP_2. Therefore, in follow-up experiments, ~0.2–0.5 μM IP_3 was used. At such a con-centration, photorelease of IP_3 at one side of the apical dome (n=20) resulted in 45% reorientation towards the side of the UV pulse and there was no visible effect on the other cells. The magnitude and response pattern was similar to PIP_2 with cells exhibiting a gradual and smooth curvature of the

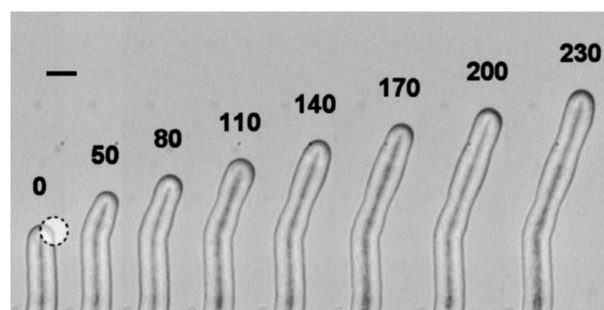

Fig. 1. Time course images of *A. umbellatus* pollen tube loaded with caged PIP_2 (~0.5–0.8 μM) and exposed to a 5 s UV pulse at the right side of the apex (circled region), immediately after time 0. The times (s) at which the images were taken are indicated next to the images. Pollen tube bent to the side of PIP_2 release; similar data was obtained for IP_3. Controls involved exposing unloaded cells to the same UV pulse which revealed no effect on the growth direction. Scale bar=10 μm.

growth axis (Fig. 1) while growth rates experienced a non-significant variation (<5%).

If, however, the photoactivation was performed in the first 40–50 μm of the pollen tube, the effects of PIP_2 and IP_3 on growth and morphology were more intense. Release of ~0.5–0.8 μM PIP_2 transiently affected apical morphology and inhibited growth rates in all cells (*n*=8). The observed responses included temporary growth arrest followed by apical swelling and recovery, suggesting a threshold concentration for this molecule and/or its end-products (for supplementary information see JXB online). Equivalent observations were made for the photorelease of ~0.2–0.5 μM caged IP_3 (*n*=5). Because mapping intracellular changes upon such responses is considerably more reliable (for supplementary information about technical details see JXB online) subsequent experiments were performed with photorelease in the first 40–50 μm of the pollen tube.

Influence of PIP_2 and IP_3 on the tip-focused $[Ca^{2+}]_c$ gradient

IP_3 is a known mobilizer of intracellular Ca^{2+} and PIP_2 its precursor. To understand the role of the two phosphoinositides in the regulation of the tip-focused $[Ca^{2+}]_c$ gradient, apical $[Ca^{2+}]_c$ was monitored while manipulating the PIP_2 and IP_3 levels.

When cells were loaded with ~0.5–0.8 μM of caged PIP_2, photolysis in the first 40–50 μm of the pollen tube (*n*=7) induced a transient $[Ca^{2+}]_c$ increase (Fig. 2A–C). This caused a transient reduction in growth rates (from $0.36\pm0.04\ \mu m\ s^{-1}$ to $0.32\pm0.05\ \mu m\ s^{-1}$) and apical bulging followed by rapid recovery (average growth rate 100 s after photolysis=$0.38\pm0.06\ \mu m\ s^{-1}$). $[Ca^{2+}]_c$ increased both in the apical and sub-apical region so the tip-focused gradient was not completely abolished. In these circumstances, growth was not totally arrested even though apical perturbations occurred. These perturbations (e.g. changes in direction of growth axis) were often accompanied by changes in the steepness of the $[Ca^{2+}]_c$ gradient (Fig. 2C, arrows).

Photoactivation of caged IP_3 (~0.2–0.5 μM) (*n*=5) was found to cause an overall $[Ca^{2+}]_c$ increase of magnitude similar to the release of PIP_2. It was, nevertheless, spatially different. With caged IP_3, the $[Ca^{2+}]_c$ increase which followed photorelease was minimum in the apex and high in the sub-apical region. In the cell illustrated in Fig. 2D–F this led to dissipation of the $[Ca^{2+}]_c$ gradient and consequent growth arrest, despite the fact that overall $[Ca^{2+}]_c$ remained elevated. On average, growth rates of the cells exposed to such stimuli decreased from $0.35\pm0.02\ \mu m\ s^{-1}$ to $0.12\pm0.06\ \mu m\ s^{-1}$ in the 100 s that followed photolysis. In subsequent phases, $[Ca^{2+}]_c$ decreased and swelling of the tube apex occurred (Fig. 2F, grey bar). Concomitantly to growth recovery, apical $[Ca^{2+}]_c$ increased and the tip-focused gradient was re-established. Controls involved exposing unloaded cells to the same UV pulse (Camacho and Malhó, 2003; Malhó and Trewavas, 1996), which revealed no effect on $[Ca^{2+}]_c$.

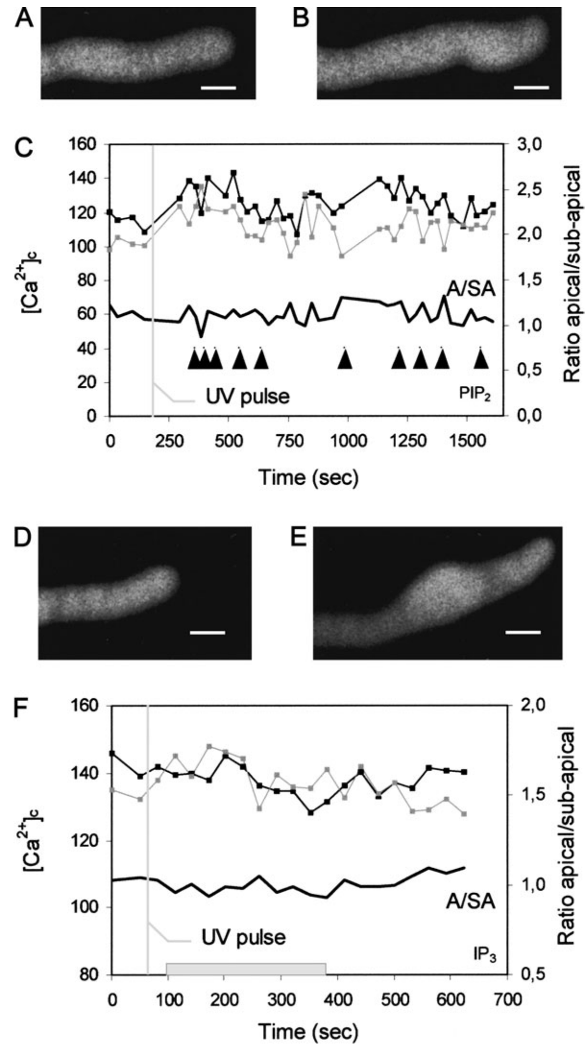

Fig. 2. Effect of photorelease of loaded PIP_2 and IP_3 on the distribution of apical $[Ca^{2+}]_c$ (A; solid line) and sub-apical $[Ca^{2+}]_c$ (SA; grey line). The solid thick trace represents the ratio A/SA; values higher than 1 indicate the existence of an apical gradient. For the sake of graph clarity, data on growth rates were not depicted here. Figure 3B and C shows the changes in growth rates induced by identical treatments in similar cells. (A–C) Photoactivation of caged PIP_2 at 150 s (indicated by the vertical line in C). Pollen tube growth was slowed down but not completely arrested. (A) and (B) are unprocessed single channel images collected at 940 s and 1130 s, respectively. Arrowheads indicate times at which reorientation occurred. Equivalent data were obtained in seven independent experiments. (D–F) Photoactivation of caged IP_3 at 50 s (indicated by the vertical line in F). Shortly after the UV pulse, growth was temporally arrested (indicated by the grey bar), but resumed at ~400 s. (D) and (E) are images collected at 50 s and 500 s, respectively. Equivalent data were obtained in five independent experiments.

PIP_2 and IP_3 differentially modulate apical secretion

It has been shown that the apical secretory machinery intersects signals from multiple signalling pathways

(Camacho and Malhó, 2003; Rato *et al.*, 2004). Thus, the effect of changing PIP_2 and IP_3 levels in pollen tubes loaded with FM 1-43 (a marker of membrane recycling) was investigated. In growing cells, this dye exhibits a tip-focused gradient that correlates with the high vesicle content in the apex (Camacho and Malhó, 2003).

As mentioned previously, cells loaded with caged PIP_2 (~0.5–0.8 μM) and exposed to a 5 s UV flash in the first 40–50 μm, showed transient reductions of growth rates, bulged at the apex and usually formed a new growth axis (Fig. 3A). This process was accompanied by an increase in apical FM 1-43 fluorescence indicating accumulation of vesicles and/or inhibition of apical secretion (Fig. 3B) (n=12). The increase in FM fluorescence averaged +23.4%, but changes up to +84.8% were recorded (SE= +19.7%). Recovery of normal growth was concomitant with a decrease in apical fluorescence intensity while fluorescence levels in the sub-apical region remained approximately uniform throughout the experiment.

The effect of IP_3, as observed before, was different from PIP_2. When cells were loaded with the same concentration used for the $[Ca^{2+}]_c$ imaging (~0.2–0.5 μM; n=11), growth was inhibited, but a decrease in FM apical fluorescence averaging −32.3% (SE= −7.5%) was recorded (Fig. 3C), suggesting higher membrane turn-over possibly through vesicle fusion and membrane recycling. Within 2–4 min pollen tubes recovered and this was concomitant with a gradual recovery of apical FM fluorescence levels and the re-establishment of the typical tip-focused gradient.

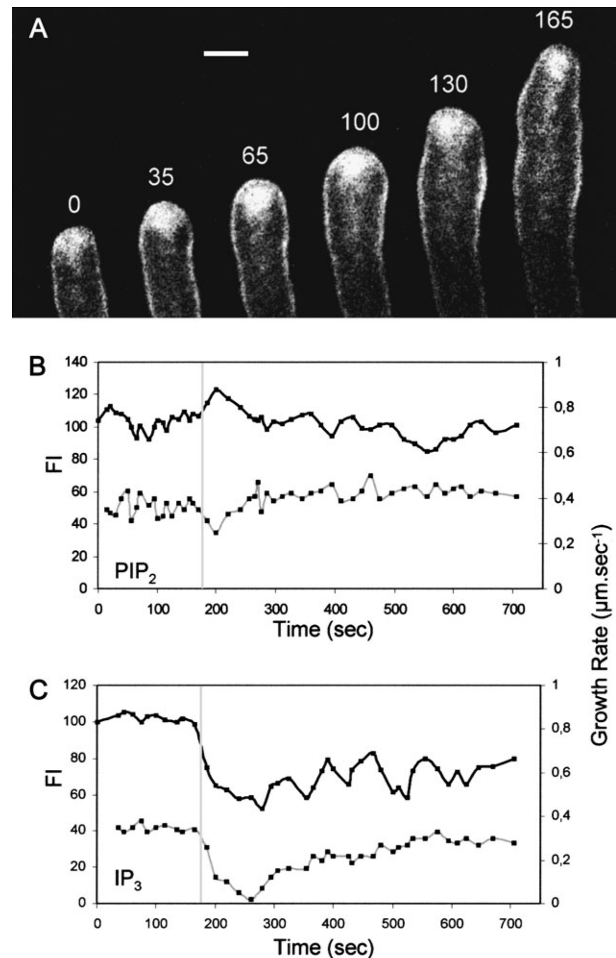

Fig. 3. Fluorescence imaging of the FM 1-43 apical staining before and after caged release of PIP_2 and IP_3. (A) Confocal time-course image series of a pollen tube loaded with caged PIP_2 and exposed to a 5 s UV pulse, immediately after time 0. The times (s) at which the images were taken are placed near the top of the pollen tubes. Cells loaded only with FM dye (without caged) showed no changes in morphology or fluorescence intensity when UV flashed (n=11; data not shown). Scale bar=10 μm. (B) Comparative analysis of FM 1-43 apical staining (black line) and pollen tube growth rate (grey line) before and after photolysis of caged PIP_2 (indicated by the vertical line). Fluorescence intensity (FI) was measured in the first 10 μm of an individual *A. umbellatus* pollen tube apex. Equivalent data were obtained in 12 independent experiments. (C) Similar to (B) but with caged IP_3. Equivalent data were obtained in 11 independent experiments.

Inhibition of PA production dissipates the tip-focused $[Ca^{2+}]_c$ gradient and inhibits membrane recycling

The data presented so far indicates that increasing the levels of PIP_2 and IP_3 had distinct effects at the cellular level. It was also observed that, for a similar concentration, IP_3 affects growth much more intensely than PIP_2. Therefore, the results can not be explained solely by a PIP_2 conversion to IP_3. Among the different targets and end-products of PIP_2, it was decided to test if the observed differences could be attributed to PA, which was recently shown to be important for pollen tube growth (Potocký *et al.*, 2003). Intracellular PA levels can be manipulated using butan-1-ol; this alcohol forms an ester with PA, phosphatidylbutanol, thus decreasing the availability of PA (for supplementary information see JXB online). Butan-2-ol, a steric isomer of butan-1-ol has no such effect and can be used as a negative control.

Butan-1-ol was found to inhibit germination, an effect that was minimized by the addition of PA (Fig. 4). Addition of 100 mM butan-1-ol to germinating pollen grains decreased the germination% from 75.2% (control; Fig. 4A; SE=6.6%) to 21.8% (Fig. 4B; SE=4.5%). This value increased to 65.6% (SE=8.2%) in the presence of 100 mM butan-1-ol and 1.0 μM PA (Fig. 4C). If added to growing pollen tubes (n=125), butan-1-ol (100 mM) caused growth arrest and loss of apical polarity-swelling (82.4%; SE=11.2%). The effect was fully reversible as confirmed by butan-1-ol washout upon which all pollen tubes recovered tip growth within 20–45 min (Fig. 5A). This time interval was significantly shortened (to 5–10 min) by the addition of PA (1 μM). Loss of polarity was not observed after application of butan-2-ol. The tip-focused $[Ca^{2+}]_c$ gradient was found to be rapidly abolished by butan-1-ol (Fig. 5B; n=8), concomitantly to growth arrest, indicating the importance of PA for apical growth. On average, growth rates of

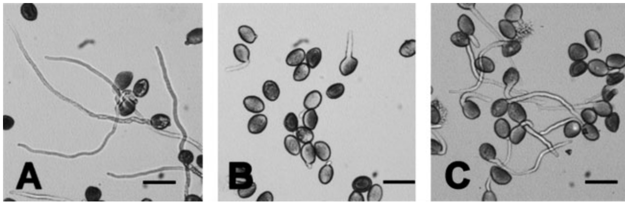

Fig. 4. Effect of butan-1-ol on pollen germination and rescue effect of PA. Bar = 100 μm. (A) Control pollen tubes 40 min after beginning of germination. (B) Pollen germination in the presence of 100 mM butan-1-ol is severely reduced and emergence of pollen tubes retarded. (C) Pollen germination in the presence of 100 mM butan-1-ol and later addition of 1 μM PA. Germination% and growth rate are partially restored.

the cells exposed to such stimuli decreased from 0.33± 0.04 μm s^{-1} to 0.03±0.04 μm s^{-1} in the 100 s that followed photolysis. Indeed, any putative interference with the production of PA via PLD or PLC, together with the DAG-kinase, caused non-polar growth (for supplementary information see JXB online). Thus, PA production could be one of the causes for the different effects induced by PIP_2 and IP_3. During the period of growth inhibition, $[Ca^{2+}]_c$ in the apical and sub-apical region remained approximately uniform. In the swelling phase that precedes recovery (Fig. 5B, grey bar), $[Ca^{2+}]_c$ in the apex reached minimum values before re-establishment of a 'new' tip-focused gradient.

Inhibition of PA production by butan-1-ol also had significant effects on apical secretion (Fig. 5C, D; *n*=6). The alcohol caused a transient decrease in apical FM fluorescence indicating the reduction of vesicles in the apex. The decrease averaged −24.5%, but reduction up to −65.2% was observed (SE= −15.4%). However, and unlike other stimuli, fluorescence increased in the sub-apical region (Fig. 5C); fluorescence in the apex only recovered when growth resumed (Fig. 5C, D; for supplementary information see JXB online).

PA is important to maintain ultrastructural polarity

In animal cells, PA was shown to be important, not only for membrane curvature and vesicular trafficking but also for cytoskeletal dynamics (Kooijman *et al.*, 2003) and therefore affecting organelle positioning. It was found that the reduction of PA levels does not affect the presence of microfilaments *per se* but significantly changes its arrangement (for supplementary information see JXB online) so its effect was investigated in the ultrastructural organization of the pollen tube. Growing *Agapanthus* pollen tubes exhibit a typical zonation similar to many other species: an apical region rich in secretory vesicles followed by a zone with many mitochondria, ER, and dictyosomes (Fig. 6A). Pollen tubes treated for 30 min with butan-1-ol (100 mM) showed no apical accumulation of secretory vesicles confirming this study's observations with the FM dye. Instead, larger organelles like mitochondria and small vacuoles penetrated

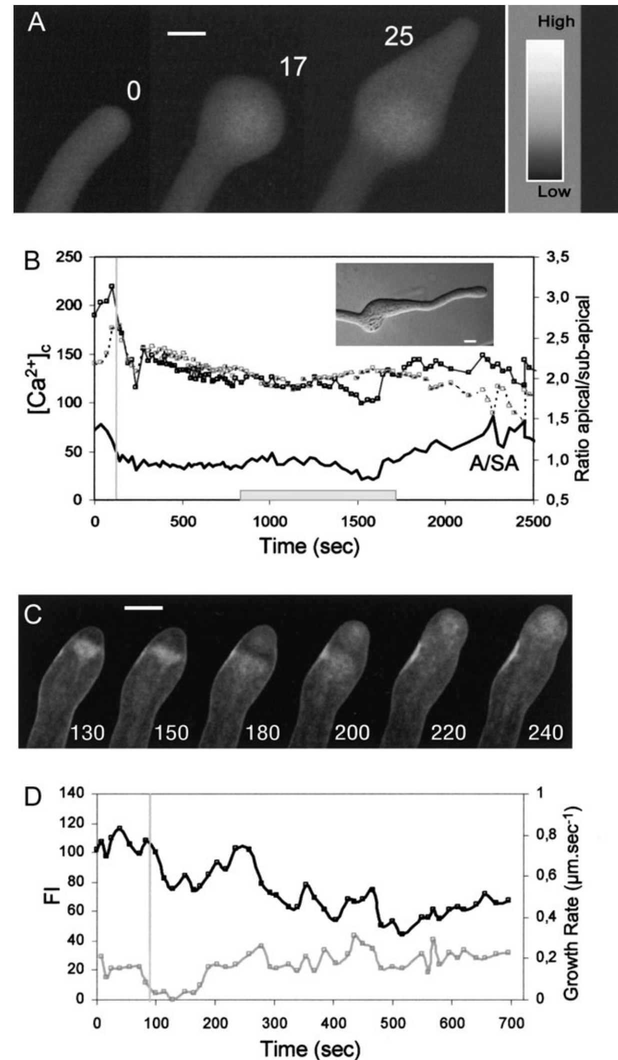

Fig. 5. Effect of butan-1-ol on apical secretion and $[Ca^{2+}]_c$. (A) Confocal time-course images of a growing pollen tube loaded with Calcium Green-1/Rhodamine B before treatment with 100 mM of butan-1-ol for 5 min. Growth was arrested (2–20 min) but recovers afterwards. (B) Changes in apical (A; solid line, black boxes) and sub-apical $[Ca^{2+}]_c$ (SA; dotted line, grey boxes) induced by butan-1-ol (added at the vertical line, 120 s) in another cell. The solid thick trace represents the ratio A/SA; values higher than 1 indicate the existence of a gradient. For the sake of clarity, data on growth rates were not depicted here. Figure 5D shows the changes in growth rates induced by identical treatment. Butan-1-ol dissipates the gradient leading to growth arrest. Pollen tube recovery is initiated by apical swelling (indicated by the grey bar) and re-establishment of the $[Ca^{2+}]_c$ gradient. Equivalent data were obtained in eight independent experiments. Insert is a DIC image of a pollen tube approximately 9 min after butan-1-ol washout. (C) Confocal time-course images of a pollen tube loaded with FM 1-43 after 1 min treatment with butan-1-ol. (D) Numerical representation of changes in FM 1-43 apical staining (black line) and growth rate (grey line) induced by butan-1-ol (added at vertical line) in the pollen tube depicted in 4C. Equivalent data were obtained in six independent experiments.

the bulging apex (Fig. 6B). These observations confirm the importance of PA in plant vesicular trafficking and, consequently, in the establishment and maintenance of polar growth.

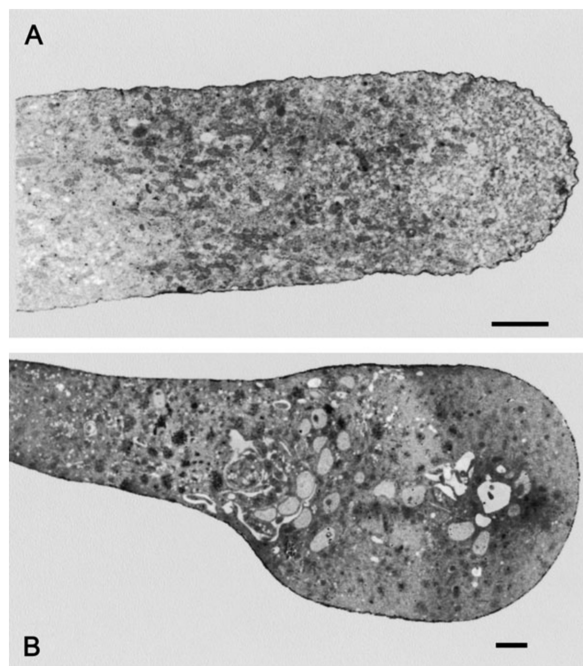

Fig. 6. Effect of butan-1-ol on apical pollen tube ultrastructure. (A) TEM image of control pollen tube. Note the dense packing of secretory vesicles in the very tip of the growing tube followed by a zone with abundant mitochondria. (B) TEM image of pollen tube treated with butan-1-ol 100 mM for 30 min and fixed immediately after washout. No secretory vesicles are visible in the very tip of the bulging tube and the clear zone observed in untreated growing pollen tubes is absent.

Discussion

Intracellular changes in PIP_2 and IP_3 modify pollen tube growth rate and axis orientation

Phosphoinositides and phospholipids are key players in plant cell signalling (Munnik, 2001). Still, when compared with what is known in animal cells, this is only the start of understanding the enormous complexity and list of characters involved. Pollen tubes are an ideal system to investigate signalling mechanisms and it was previously found that an increase in the levels of IP_3, either in the nuclear or sub-apical region, resulted in the reorientation of the cell growth axis and a transient increase in $[Ca^{2+}]_c$ (Malhó, 1998). It was also observed that pronounced changes in the levels of apical IP_3 resulted in tip bursting, suggesting a fine regulation of homeostasis. Those observations were extended by releasing different concentrations of caged-IP_3 and the effect on reorientation and growth rate was tested. *In vivo*, IP_3 arises from the hydrolysis of its precursor, PIP_2, and thus the effect of releasing this molecule was also compared.

Photorelease of ~0.5–0.8 μM PIP_2 or ~0.2–0.5 μM IP_3 on one side of the apical dome only caused reorientation towards the side of release. This shows their involvement in the intracellular mechanism controlling cell guidance, an hypothesis already reported for axonal growth (Dickson and Senti, 2002). The cells changing growth direction exhibited a smooth curvature of the growth axis while growth rates experienced a non-significant variation. This contrasts with the photorelease in the first 40–50 μm of the cell apex where reorientation was preceded by transient growth arrest and/or apical deformation. Interestingly, at lower concentrations [~0.2–0.5 μM and ~0.1–0.2 μM for PIP_2 and IP_3, respectively], photolysis of both probes was found to promote slight increases in growth rates (for supplementary information see JXB online). These observations can be explained by the effect of both molecules already reported in the literature: for example, PIP_2 is required for the regulation of Ca^{2+} channels (Wu *et al.*, 2002) and microfilament scaffolding (Raucher *et al.*, 2000); IP_3 is known to play a key role in Ca^{2+} mobilization (DeWald *et al.*, 2001) and regulation of cAMP levels (Bruce *et al.*, 2002). This indicates that optimum growth depends on a tight regulation of phosphoinositide levels. Similar observations have been made for protein kinase activity (Moutinho *et al.*, 1998), cAMP (Moutinho *et al.*, 2001), $[Ca^{2+}]_c$ and GTPase activity (Camacho and Malhó, 2003) thus suggesting a close association between the different signalling pathways (Malhó and Camacho, 2004). Crossing the 'concentration thresholds' for these molecules does not necessarily represent an inhibitory effect; they all have been found to be associated with reorientation of the growth axis and/or perception of extracellular cues. Therefore, concentrations were used which induce measurable changes in growth morphology, direction, and intracellular dynamics; they are invariably over the threshold for growth stimulation but are representative of physiological responses and provide meaningful data.

As reported for animal cells (Bird *et al.*, 1992), it was also found that, for a similar intracellular concentration, the effect of releasing IP_3 was much more dramatic than the equivalent release of PIP_2. This indicates that cells tolerate different concentrations of the two phosphoinositides, which prompted an investigation into the consequences of this fact. The studies focused on the dynamics of $[Ca^{2+}]_c$ and membrane secretion because of their importance during the reorientation process (Camacho and Malhó, 2003; Rato *et al.*, 2004).

PIP_2 and IP_3 modulate the tip-focused $[Ca^{2+}]_c$ gradient and apical secretion

PIP_2, the precursor of several signalling molecules, is itself also used by cells to signal to membrane-associated proteins. In addition, PIP_2 anchors numerous molecules and the cytoskeleton to the plasma membrane, and its metabolism is closely connected to membrane trafficking (Stevenson *et al.*, 2000). This phosphoinositide has thus been implicated in a myriad of functions (for an extensive description see Stevenson *et al.*, 2000) although in most cases it is not clear if the response observed is a direct action of PIP_2 or results from a signalling cascade. It was observed that after photorelease of caged PIP_2 in the first 40–50 μm of the pollen tube, $[Ca^{2+}]_c$ increased both in the

apical and sub-apical regions. Apical morphology was affected, growth rates decreased (but not to a complete halt) and the $[Ca^{2+}]_c$ gradient was not totally dissipated. Using this methodology the source of the $[Ca^{2+}]_c$ increase can not be established. Nevertheless, maintenance of a gradient and, concomitantly growth, has been observed to require apical Ca^{2+} influx (Holdaway-Clarke and Hepler, 2003), thus this hypothesis is favoured.

Release of IP_3 also resulted in a $[Ca^{2+}]_c$ increase, as previously reported (Malhó, 1998). However, it was spatially distinct from the PIP_2 effect, with $[Ca^{2+}]_c$ increasing mainly in the sub-apical region. Consequently, the tip-focused $[Ca^{2+}]_c$ gradient was disrupted and growth arrested. The sub-apex of the pollen tube is an area rich in endoplasmic reticulum profiles (Holdaway-Clarke and Hepler, 2003), which have been reported to bind IP_3 strongly (Martinec *et al.*, 2000). This suggests that the IP_3-induced Ca^{2+} increase results mainly from the activation of the intracellular store. IP_3 levels may then play a preponderant role in the regulation of sub-apical processes (e.g. actin dynamics), a possibility already discussed by Malhó and Camacho (2004).

It is well known that high levels of Ca^{2+} are associated with secretion (Battey *et al.*, 1999). This holds for pollen tubes where membrane fusion and recycling was reported to be higher in the side of the apical dome to which the cell bent and to correlate directly with changes in apical $[Ca^{2+}]_c$ (Camacho and Malhó, 2003). Interestingly, although release from both phosphoinositides caused transient $[Ca^{2+}]_c$ rises, their effect on membrane secretion was markedly different. Release of PIP_2 caused a slight increase in apical FM fluorescence suggesting a decrease in apical secretion. This can either represent an increase in the rate of vesicle migration to the apex and/or higher membrane recycling. By contrast, release of IP_3 caused a decrease in FM apical fluorescence which was interpreted as the inhibition of new vesicle production and/or a higher rate of apical vesicle fusion.

These observations indicate that these data can not be explained solely by a PIP_2 hydrolysis to IP_3 and Ca^{2+} changes. Different targets and/or end-products of their conversion must be considered.

PA levels modulate the tip-focused $[Ca^{2+}]_c$ gradient and membrane secretion

Among the several candidates to explain a differential effect between modulation of intracellular $[PIP_2]$ and $[IP_3]$ is PA. This phospholipid can be generated from DAG through DAG kinase or through PLD activity in a PIP_2-dependent process (Munnik, 2001). The importance of PA for polar growth has recently been demonstrated (Potocký *et al.*, 2003; Zonia and Munnik, 2004; S Lisboa *et al.*, unpublished data) and adds to other reports about lipid signalling in plant reproduction (Wolters-Arts *et al.*, 1998; Park *et al.*, 2000; Lalanne *et al.*, 2004).

Using butan-1-ol, the intracellular levels of PA could be manipulated while its effect on $[Ca^{2+}]_c$ and membrane trafficking could be imaged. Butan-1-ol forms an ester with PA (phosphatidylbutanol) thus decreasing the concentration of available PA. As reported for other species (Potocký *et al.*, 2003), it was found that butan-1-ol caused reversible loss of polarity and the specificity of the response was confirmed by treatment with butan-2-ol (an isomer of butan-1-ol). Furthermore, it was also observed that the magnitude/duration of the butan-1-ol effect could be diminished by the addition of extracellular PA. One of the effects of decreasing the intracellular concentration of PA was the immediate dissipation of the tip-focused $[Ca^{2+}]_c$ gradient. This is not a surprising observation *per se*. Any stimuli leading to growth arrest has been found to disrupt the gradient (Holdaway-Clarke and Hepler, 2003; Malhó and Camacho, 2004), either because of a direct effect on Ca^{2+} fluxes or as a consequence from some other structural inhibition. However, the dynamics recorded after butan-1-ol addition was different from what was observed after photolysis of the caged-phosphoinositides. Reduction of the PA levels caused an overall reduction in $[Ca^{2+}]_c$, but apical $[Ca^{2+}]_c$ started to rise still in the swelling phase of recovery. PA (endogenous as well as exogenous) was reported to stimulate the translocation of Ca^{2+} across cell membranes (Ohsako and Deguchi, 1981) and Zonia and Munnik (2004) found that PA increases during pollen tube swelling, observations that support these data.

The possibility that PA acts as a regulator of Ca^{2+} fluxes is interesting but, if no other target is considered, it fails to explain these data fully. Since a well-known effect of PA is the promotion of secretory vesicle formation (Sweeney *et al.*, 2002; Kooijman *et al.*, 2003), the dynamics of membrane trafficking in the apex of pollen tubes submitted to the butan-1-ol treatment were analysed. Fluorescence measurements with the FM 1-43 dye suggested that reduction of PA levels caused a strong inhibition of apical membrane recycling (via endocytosis) and further supply of vesicles to the apex. Vesicles (and possibly other Golgi-derived structures labelled by the FM dye) accumulated in the sub-apical region and further movement into the apex was blocked. Therefore, PA is suggested to play an important role in apical vesicle dynamics.

Reduced PA induces non-polar ultrastructural organization

The depletion of FM fluorescence in the apex of butan-1-ol treated cells, together with the ultrastructural data, contrasts with the strong signal in the sub-apical region and indicates that, in reduced PA, polar vesicle transport is inhibited. In addition to a role in membrane curvature, PA has been reported to regulate microfilament polymerization and to play a role in membrane transport (Bi *et al.*, 1997; Kooijman *et al.*, 2003). It was observed that the bulging

effect at the apex induced by reduction of PA levels was accompanied by the formation of thick but non-directional microfilaments. This suggests that PA participates in the correct anchoring and positioning of actin microfilaments (for supplementary information and an additional discussion see JXB online). Phospholipids and phosphoinositides could thus provide a link between signalling pathways and structural aspects of apical growth.

These multiple but co-ordinated effects of PA might explain the different responses induced by photolysis of caged PIP_2 or IP_3. An increase in PIP_2 is likely to result in its hydrolysis leading to increased levels of IP_3 and PA. The effects of PA on membrane curvature are enhanced by Ca^{2+} (Kooijman *et al.*, 2003) thus, simultaneously to the $[Ca^{2+}]_c$ rise, an increase in membrane turnover and actin-dependent trafficking might occur. This promotes a faster return to resting conditions without complete disruption of apical growth. By contrast, increasing IP_3 levels leads to a rise in $[Ca^{2+}]_c$ and increased exocytosis, but not to equivalent membrane recycling. In addition, IP_3 turnover after photolysis is slow (Franklin-Tong *et al.*, 1996) and can result in prolonged inhibition of PIP_2 hydrolysis and decrease in PA production. As a consequence, homeostasis is severely perturbed.

These data show the involvement of PIP_2, IP_3, and PA in the regulation of polarized growth and reorientation through a combined interaction at multiple levels (ionic fluxes, secretion, ultrastructure) thus placing phosphoinositides and lipids as key regulators of tip growth. Further characterization of these pathways and the extent of cross-talk with other signalling pathways is now required. GTPases are undoubtedly involved in this process (Kost *et al.*, 1999) but also, most likely, calmodulin and cAMP (Rato *et al.*, 2004). The identification of their targets and patterns of expression/activity, are challenging but essential tasks to understand how such a diversity of molecules acts together to elicit physiological responses.

Supplementary data

Supplementary information can be found at JXB online.

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