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SHORT COMMUNICATION

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Role of 1,4,5-inositol triphosphate-induced Ca^{2+} release in pollen tube orientation

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Abstract Pollen tube reorientation is a dynamic cellular event crucial for successful fertilization. Previously, it was shown that reorientation is preceded by an asymmetric increase of cytosolic free calcium ($[Ca^{2+}]_c$) in the side of the apex to which the cell will bend. In order to find the targets for this signal transduction pathway, the effects of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] in the reorientation process were analyzed. Ins(1,4,5)P_3 was artificially increased in different cell domains by localized photoactivation of caged Ins(1,4,5)P_3 and its effects on $[Ca^{2+}]_c$ monitored by ion confocal microscopy. It was found that photolysis of caged Ins(1,4,5)P_3 in the nuclear or subapical region resulted in a transient increase in $[Ca^{2+}]_c$ and reorientation of the growth axis, while photolysis in the apex frequently resulted in disturbed growth or tip bursting. Perfusion of the cells with the Ins(1,4,5)P_3 receptor blocker heparin prior to photoactivation inhibited the increase in $[Ca^{2+}]_c$ and no reorientation was observed. Ca^{2+} release from Ins(1,4,5)P_3-dependent stores localized in the shank of the tube thus seems to be part of the signal transduction pathway that controls tube guidance, although not the primary stimulus leading to reorientation.

Key words Calcium · Ins(1,4,5)P_3-induced-Ca^{2+}-release · Pollen tube · *Agapanthus umbellatus*

Introduction

In previous work (Malhó et al. 1994, 1995; Malhó and Trewavas 1996) it has been shown that the distribution of $[Ca^{2+}]_c$ in the apical region and Ca^{2+} influx by an asymmetric activity of Ca^{2+} channels have crucial roles in tube guidance. However, how cells interpret this information is not known.

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In non-excitable cells, the predominant mechanism for Ca^{2+} release is triggered by inositol 1,4,5-triphosphate [Ins(1,4,5)P_3], a diffusible messenger (Berridge 1993). In contrast, excitable cells rely on a Ca^{2+}-induced Ca^{2+}-release mechanism triggered by an influx of Ca^{2+} from voltage-dependent Ca^{2+} channels. Although signaling in both types of cells is regulated by plasma membrane Ca^{2+} channels, these channels seem to be different in each case. However, recent reports have revealed unexpected similarities in these two Ca^{2+} signaling mechanisms (De Waard et al. 1992; Putney 1993). In plants, Ins(1,4,5)P_3 has been shown to release Ca^{2+} from a number of cell fractions (Drøbak and Ferguson 1985) and recent studies on the self-incompatibility mechanisms of poppy (Franklin-Tong et al. 1996) have characterized the activity of a Ca^{2+}-activated phospholipase C. Thus, it was decided to examine the possible role of Ins(1,4,5)P_3 in the reorientation mechanism of pollen tubes. This was achieved by loading a UV-sensitive caged Ins(1,4,5)P_3 into the cells and releasing it in spatially restricted areas (Malhó and Trewavas 1996).

Material and methods

Pollen tubes of *Agapanthus umbellatus* were grown in vitro as described previously (Malhó et al. 1994; Malhó and Trewavas 1996) and microinjected with the free acid of the Ca^{2+}-sensitive dye Calcium Green-1 (Molecular Probes, Eugene, Ore.). Changes in $[Ca^{2+}]_c$ were imaged by confocal microscopy; detailed analysis and critical assessment of this procedure can be found in Malhó and Trewavas (1996).

$[Ca^{2+}]_c$ values were artificially manipulated by photoactivating caged Ins(1,4,5)P_3 (Calbiochem, Nottingham, UK) in different cytological regions of the pollen tube using a flash photolysis system. This system allows photolysis in circular areas of about 80–95 μm^2 (Fig. 1); detailed analysis of its characteristics (resolution, efficiency and limitations) can be found in Malhó and Trewavas (1996).

Caged Ins(1,4,5)P_3 was microinjected (100–500 μM) together with Calcium Green-1 using the ionophoretic conditions described in Malhó and Trewavas (1996) and with $[Ca^{2+}]_c$ measured before and after photoactivation. The concentration of Ins(1,4,5)P_3 released was estimated by the caged fluorescein method (Malhó and Trewavas 1996). Pollen tubes were also loaded with caged-

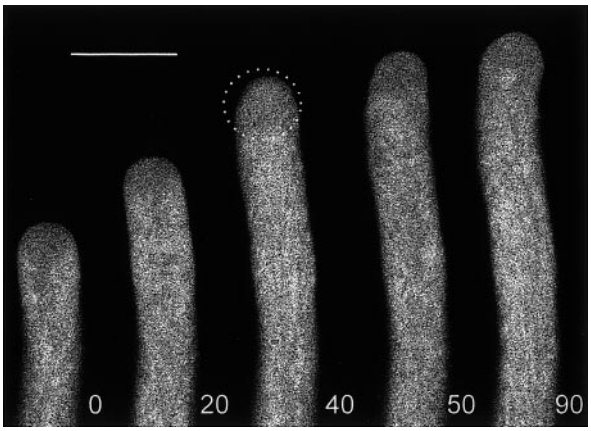

Fig. 1 Confocal time course series of a release of caged $Ins(1,4,5)P_3$ in the apical region (indicated by *circle*) at ~40 s. An increase in $Ins(1,4,5)P_3$ levels in the apex was not followed by an equivalent increase in $[Ca^{2+}]_c$ and soon after the release the tube ceased its growth and did not recover. The times (s) at which images were taken are shown adjacent to the right of the growing tubes. *Bar* 20 μm

$Ins(1,4,5)P_3$, Calcium Green-1 and heparin (1% of a 140 units mg^{-1} stock solution; Sigma, Dorset, UK) and $[Ca^{2+}]_c$ imaged upon photolysis. Details of the experimental procedure and criteria used to establish the success of microinjection can be found in Malhó et al. (1994). Numerical data presentation follows the criteria defined in Malhó and Trewavas (1996).

Results

Effect of localized photoactivation of caged $Ins(1,4,5)P_3$ on pollen tube $[Ca^{2+}]_c$ and orientation

Photoactivation of caged $Ins(1,4,5)P_3$ was performed in different regions of the pollen tube as reported earlier (Malhó and Trewavas 1996). These were the nuclear region, 30–50 μm behind the tip; the subapical region, 15–30 μm behind the tip; and the apical dome, 0–15 μm from the tip. Quantification of $[Ca^{2+}]_c$ imaging with Calcium Green-1 is given in Fig. 2. Calcium Green-1 is a non-ratio dye and although fluorescence intensity is directly proportional to changes in the levels of $[Ca^{2+}]_c$ it does not allow precise quantification. Therefore, the variation in dye fluorescence should be compared only within each cytological region. Estimates of $[Ca^{2+}]_c$ changes presented in this work were calculated based on previous measurements in *A. umbellatus* using the ratioable dye Indo-1 (Malhó et al. 1994, 1995) and should be regarded only as rough approximations to true values. The concentration of $Ins(1,4,5)P_3$ released was estimated according to our caged fluorescein calibration method (Malhó and Trewavas 1996) to be 500–700 nM in the nuclear region and 200–400 nM in the apical and subapical regions. Higher values were found to result in extremely disturbing effects for pollen tubes, namely complete arrest of growth and tip bursting.

When the photolysis was carried out in the nuclear region (n=12; eight tubes showing reorientation) an immediate increase in $[Ca^{2+}]_c$ was observed in this area (Fig. 2A). This effect was similar to that observed with the release of caged Ca^{2+} in the same region (Malhó and Trewavas 1996). First, an elevation in $[Ca^{2+}]_c$ level reaching about 0.9–1 μM spreads toward other regions in a slow wave-like fashion (~70 μm min^{-1}). Second, growth rates decline when the elevation reaches the tip. Third, there is random reorientation of the growth axis upon recovery. The similarities in these two sets of data clearly suggest that, in vivo, $[Ca^{2+}]_c$ increases result from opening of $Ins(1,4,5)P_3$-dependent intracellular stores.

When photolysis of caged $Ins(1,4,5)P_3$ was performed in the subapical region, the effects were again similar to those from photolysis of caged Ca^{2+}. In the 16 tubes where the experimental procedure was carried out, $[Ca^{2+}]_c$ elevations reaching 600–700 nM were observed (Fig. 2B). Two of these tubes stopped growing and did not recover, three tubes recovered in the same direction, and 11 tubes showed reorientation of the growth axis. If cells are loaded with a higher concentration of caged $Ins(1,4,5)P_3$ by increasing either the concentration in the needle or the loading period, the increase in $[Ca^{2+}]_c$ could reach values above 1 μM. When this happened, pollen tubes did not recover (n=9) and occasionally tip bursting was observed (n=3).

The main differences from the data obtained with caged Ca^{2+} (Malhó and Trewavas 1996) were observed when $Ins(1,4,5)P_3$ was released in the apical region. In ten tubes where photolysis was achieved in the overall apical region, four burst 30–60 s after release, four stopped growing and did not recover (Fig. 1), and the remaining two recovered with a new growth axis. When release was performed only in the left side of the apical region by focusing the UV beam on the edge of the pollen tube apex (n=7), two cells showed a transient decrease in growth rate but no visible reorientation, while the remaining five exhibited curvature of the growth axis (Fig. 2C). However, the angle of curvature never exceeded 35° and, in most cases (n=4), growth returned to the previous direction within 1 min. In all cases, these effects were accompanied by an elevated level of $[Ca^{2+}]_c$.

Effect of heparin on pollen tube $[Ca^{2+}]_c$ and orientation

To test the extent of a possible role for $Ins(1,4,5)P_3$ in the reorientation mechanism, two questions were addressed: (1) are the observations described here due to a direct effect of $Ins(1,4,5)P_3$ on Ca^{2+} metabolism and (2) is $Ins(1,4,5)P_3$ (and the inositol phosphate pathway in general) primarily involved in the reorientation mechanisms? A recent report on poppy pollen tubes (Franklin-Tong et al. 1996) suggested that Ca^{2+} release is $Ins(1,4,5)P_3$-dependent. However, in this work the author did not examine localized photolysis so no conclusions can be drawn about the possible localization and role of $Ins(1,4,5)P_3$ receptors.

Both of the questions were addressed with the use of heparin. Heparin is the most specific inhibitor of the in-

tracellular Ins(1,4,5)P_3 receptor currently known (Kobayashi et al. 1988) and a similar functional use was demonstrated in plant cells (Brosnan and Sanders 1990; Franklin-Tong et al. 1996). Pollen tubes loaded with heparin were used for photoactivation of caged Ins(1,4,5)P_3 as described in Materials and methods (n=10). In all cells tested the resulting $[Ca^{2+}]_c$ transient change was strongly but not totally inhibited (Fig. 2D). Growth rates declined only temporarily and no reorientation was observed. Total inhibition of the $[Ca^{2+}]_c$ transient change could be achieved by increasing the concentration of heparin loaded into the cell. However, this procedure also led to the inhibition of tube growth, precluding any further analysis. Furthermore, it was observed that tubes loaded with heparin and exposed to an external electrical field of 5 Vcm^{-1} failed to reorient and exhibited a $[Ca^{2+}]_c$ increase restricted to the tip region (Fig. 3). This con-

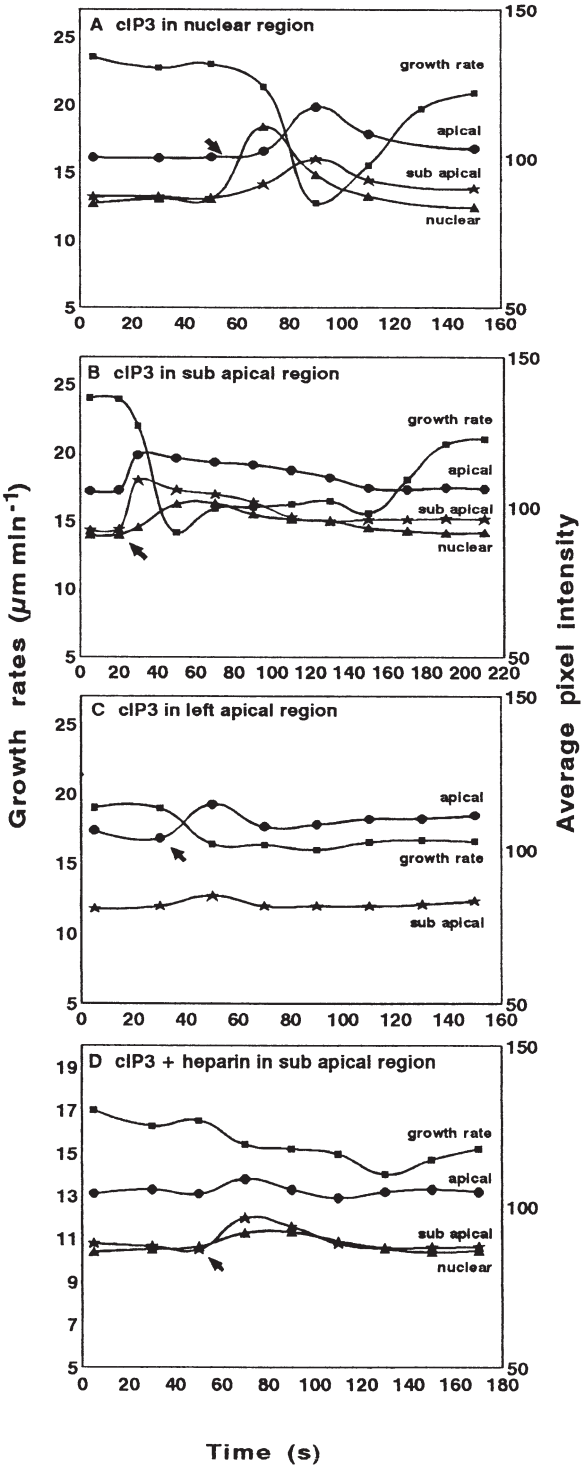

Fig. 2A–D Effect of UV photolysis of loaded caged Ins(1,4,5)P_3 in different regions of a growing pollen tube on the distribution of $[Ca^{2+}]_c$. As a control, tubes loaded with Calcium Green-1 were exposed to similar pulses of UV light. This treatment had no detectable effect on $[Ca^{2+}]_c$, growth rate or reorientation. *Filled squares* (■) represent growth rates. Calcium Green-1 pixel intensity in the apical region is represented by *filled circles* (●), in the subapical region by *stars* (☆) and in the nuclear region by *filled triangles* (▲). In order to relate pixel intensity within each region to $[Ca^{2+}]_c$ distribution, all values were multiplied by the average ratio value determined for that region with the dual wavelength dye Indo-1 (×1.3 for the apical, ×0.8 for the sub-apical, and ×0.7 for the nuclear region). *Arrows* indicate times of photoactivation. **A** Flash photolysis of loaded caged Ins(1,4,5)P_3 in the nuclear region at ≈55 s. Average pixel intensity of Calcium Green-1 was determined in the nuclear, subapical and apical regions. Random reorientation of the tube growth axis occurred after the elevation in $[Ca^{2+}]_c$ reached the tip. There is a delay between the release of Ca^{2+} in the nuclear region (*arrow*) and the increase of $[Ca^{2+}]_c$ in the subapical and apical regions. **B** Photolysis of loaded caged Ins(1,4,5)P_3 in the subapical region at ≈25 s (*arrow*) on growth rates and pixel intensity in the nuclear, subapical and apical regions. A transient increase in $[Ca^{2+}]_c$ and random reorientation were observed. **C** Photolysis of loaded caged Ins(1,4,5)P_3 in the left hemisphere of the apical region at ≈35 s (*arrow*); effects on growth rates and pixel intensity in the subapical and apical regions. A slight and short-lived reorientation of the tube growth axis followed the localized elevation in $[Ca^{2+}]_c$ in the left side of the tube apex. **D** Flash photolysis of caged Ins(1,4,5)P_3 in the subapical region (at ≈65 s; *arrow*) of a tube loaded with heparin. Average pixel intensity of Calcium Green-1 was determined in the nuclear, subapical and apical regions. Heparin inhibited almost completely the increase in $[Ca^{2+}]_c$ and no reorientation was observed

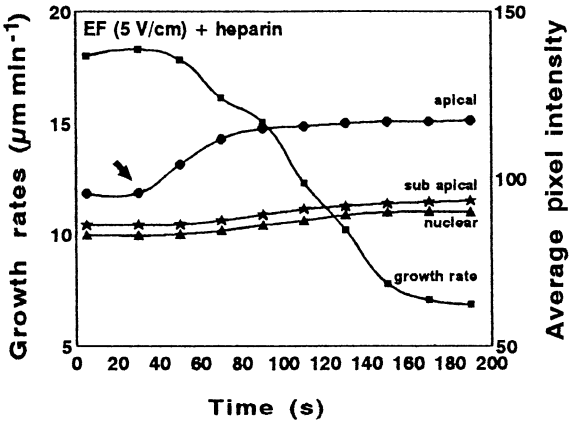

Fig. 3 Effect of a 5 V cm^{-1} external electrical field on the distribution of $[Ca^{2+}]_c$ in a growing tube loaded with heparin. The start of the electrical stimulus is indicated by an *arrow*. Average pixel intensity of Calcium Green-1 was determined in the nuclear, subapical and apical regions. Heparin inhibited almost completely the increase in $[Ca^{2+}]_c$ in nuclear and subapical regions but not in the tube apex

trasts with the effect of the electrical field per se, in which the elevation in $[Ca^{2+}]_c$ was observed throughout the 100 μm of the tube within the field of view; it is likely the elevation occurs in remote regions of the tube as well (Malhó et al. 1994). These data indicate that Ca^{2+} release can be regulated by $Ins(1,4,5)P_3$ but that $Ins(1,4,5)P_3$ receptors are active (located) mainly in the shank of the tube.

Discussion

It is clear from the data gathered so far that pollen tubes can respond to a variety of stimuli that change their growth direction. However, we must distinguish those which promote a consistent and predictable change in growth axis from those which act simply by disrupting the polarity axis, even if transiently. In vivo, it is clear that the former are the stimuli which guide the tube to the micropyle.

It was previously shown (Malhó et al. 1994, 1995; Malhó and Trewavas 1996) that reorientation is preceded by an elevation in $[Ca^{2+}]_c$ resulting from Ca^{2+} channel activity in the pollen tube plasma membrane. However, it is unlikely that the elevation recorded in distal parts of the tubes is due only to an influx of extracellular Ca^{2+}. It is plausible to assume that the initial influx of Ca^{2+} could result in subsequent releases from internal stores, a mechanism common to different cell types (Fewtrell 1993). Voltage- and/or $Ins(1,4,5)P_3$-operated channels existing in the tonoplast (Alexandre et al. 1990) or endoplasmic reticulum (Muir and Sanders 1997) could play a role in these mechanisms. Such $Ins(1,4,5)P_3$-induced-Ca^{2+}-release is a well-documented phenomenon in animal cells but not in plants. Franklin-Tong et al. (1996) observed this $Ins(1,4,5)P_3$-induced-Ca^{2+} release in pollen tubes, but to date accurate measurements of such phenomena have been obtained only in fractions corresponding functionally and structurally to the tonoplast (Canut et al. 1993).

It was found that artificially increasing the levels of $Ins(1,4,5)P_3$ in either the nuclear or subapical region resulted in random reorientation of the cell growth axis and also in a transient increase in $[Ca^{2+}]_c$. The similarities between these results and those obtained by photolysis of caged Ca^{2+} clearly suggest that, in vivo, $[Ca^{2+}]_c$ increases in the shank of the tube result from the opening of $Ins(1,4,5)P_3$-dependent intracellular stores. On the other hand, release in one half of the apical dome induced only slight or transient reorientation. A simple $Ins(1,4,5)P_3$-induced $[Ca^{2+}]_c$ elevation cannot account for this effect because the same effect is not observed with photolysis of caged Ca^{2+} (Malhó and Trewavas 1996). The results thus suggest an asymmetric functionality of $Ins(1,4,5)P_3$ receptors in pollen tubes. This is in agreement with the kinetics; $Ins(1,4,5)P_3$ receptors are profoundly affected by Ca^{2+} ions (Dawson 1997). In the nM range, increasing $[Ca^{2+}]_c$ potentiates Ca^{2+} release by $Ins(1,4,5)P_3$ to the extent that Ca^{2+} and $Ins(1,4,5)P_3$ can be regarded as co-agonists for Ca^{2+} release; in the μM range, however, Ca^{2+} is found to be inhibitory and the receptor undergoes an intrinsic inactivation when IP3 is bound. Further support for this hypothesis derives from two lines of evidence. (1) A release of high concentrations of $Ins(1,4,5)P_3$ in the apical region did not result in higher $[Ca^{2+}]_c$ elevations; in such cases tip bursting was usually the final result. Franklin-Tong et al. (1996) found that $Ins(1,4,5)P_3$ turnover after photolysis is slow and can result in prolonged inhibition of PIC and disturbance of cell homeostasis. (2) Heparin also inhibited the electrical field-induced reorientation. Imaging of Ca^{2+} under these conditions showed that the typical elevation throughout the shank of the tube (Malhó et al. 1994) no longer occurred. Instead, a localized elevation in the tip region was found. Petersen and Berridge (1994) showed that injection of heparin completely blocked responses to flash photolysis of caged $Ins(1,4,5)P_3$ but had no apparent effect on Ca^{2+} entry. In the work reported here, heparin incompletely inhibited the Ca^{2+} elevation triggered by $Ins(1,4,5)P_3$. This can be interpreted to be a direct effect of $Ins(1,4,5)P_3$ on Ca^{2+} channels through a mechanism similar to that which occurs in cerebellar granule neurons (De Waard et al. 1992). An alternative, and simpler explanation, is that the highest concentration of heparin compatible with growth was not high enough to completely block all $Ins(1,4,5)P_3$ receptors.

In conclusion, $Ins(1,4,5)P_3$ binding does not seem to be required for activation of Ca^{2+} entry, one of the primary events leading to reorientation. Instead, $Ins(1,4,5)P_3$-induced-Ca^{2+}-release seems to play a vital role in the transduction of the signal to the body of the tube.

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