The Plant Cell, Vol. 8, 1305–1321, August 1996

Growth of Pollen Tubes of *Papaver rhoeas* Is Regulated by a Slow-Moving Calcium Wave Propagated by Inositol 1,4,5-Trisphosphate

Vernonica E. Franklin-Tong,[a,1] Bjørn K. Drøbak,[b] Andrew C. Allan,[c,2] Peter A. C. Watkins,[b] and Anthony J. Trewavas[c]

[a] Wolfson Laboratory for Plant Molecular Biology, School of Biological Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

[b] Department of Cell Biology, John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom

[c] Molecular Signaling Group, Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JH, United Kingdom

A signaling role for cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in regulating *Papaver rhoeas* pollen tube growth during the self-incompatibility response has been demonstrated previously. In this article, we investigate the involvement of the phosphoinositide signal transduction pathway in Ca^{2+}-mediated pollen tube inhibition. We demonstrate that *P. rhoeas* pollen tubes have a Ca^{2+}-dependent polyphosphoinositide-specific phospholipase C activity that is inhibited by neomycin. $[Ca^{2+}]_i$ imaging after photolysis of caged inositol (1,4,5)-trisphosphate ($Ins[1,4,5]P_3$) in pollen tubes demonstrated that $Ins(1,4,5)P_3$ could induce Ca^{2+} release, which was inhibited by heparin and neomycin. Mastoparan, which stimulated $Ins(1,4,5)P_3$ production, also induced a rapid increase in Ca^{2+}, which was inhibited by neomycin. These data provide direct evidence for the involvement of a functional phosphoinositide signal-transducing system in the regulation of pollen tube growth. We suggest that the observed Ca^{2+} increases are mediated, at least in part, by $Ins(1,4,5)P_3$-induced Ca^{2+} release. Furthermore, we provide data suggesting that Ca^{2+} waves, which have not previously been reported in plant cells, can be induced in pollen tubes.

INTRODUCTION

Pollination in flowering plants results in the transfer of pollen from the anther to the stigma. Subsequent germination and pollen tube growth convey the male gametes to the ovule to effect fertilization and the formation of seed. Growing pollen tubes have become an attractive model system for experimental investigations of polarized growth as well as fundamental studies on the control of fertility and reproduction. It is now well established that cytosolic free calcium ($[Ca^{2+}]_i$) is involved as a second messenger in many signal transduction processes in plants (Trewavas and Gilroy, 1991; Gilroy et al., 1993; Bush, 1995), and it is likely that it is also involved in the regulation of pollen tube growth. The dependence of pollen tube growth on exogenous calcium has long been known (Brewbaker and Kwack, 1963; Jaffe et al., 1975; Picton and Steer, 1983; Reiss and Herth, 1985). A number of studies have also established that growing pollen tubes have a tip-based gradient of $[Ca^{2+}]_i$ (Obermeyer and Weisenseel, 1991; Rathore et al., 1991; Miller et al., 1992; Malhó et al., 1994; Pierson et al., 1994), which results from an apical concentration of open calcium channels (Malhó et al., 1995). When growth ceases, these channels close and the Ca^{2+} gradient dissipates (Pierson et al., 1994; Malhó et al., 1995), and the elimination of the gradient by calcium chelators stops further growth (Pierson et al., 1994). The precise function of this tip-based Ca^{2+} gradient remains unclear, but one possibility is that it is involved in the regulation of secretion of vesicles in the apical dome. $[Ca^{2+}]_i$ is also likely to be involved in reorientation of pollen tube growth (Malhó et al., 1994).

We established previously that self-incompatibility responses in pollen of *Papaver rhoeas* are mediated by a signal transduction pathway involving $[Ca^{2+}]_i$ (Franklin-Tong et al., 1993, 1995). Self-incompatibility is a mechanism controlled by genes at the *S* locus, which regulates the acceptance or rejection of pollen on the pistil. Treatment of pollen tubes with incompatible S proteins resulted in increases in $[Ca^{2+}]_i$, which was followed by a cessation of pollen tube growth (Franklin-Tong et al., 1993, 1995). Treatment with compatible extracts or with heat-denatured, incompatible proteins did not result in any change in $[Ca^{2+}]_i$, and pollen tube growth continued normally. Involvement of $[Ca^{2+}]_i$ in the regulation of *P. rhoeas* pollen tube growth was further demonstrated by photolysis of nitr-5 (caged calcium). The resultant increases in $[Ca^{2+}]_i$ caused

[1] To whom correspondence should be addressed.

[2] Current address: Department of Plant Genetics, Weizmann Institute of Science, P.O. Box 26, Rehovot 76100, Israel.

inhibition of growth. More recently, we identified Ca^{2+}-dependent increases in protein phosphorylation specifically triggered by the incompatible response (Rudd et al., 1996), which implicates protein kinase involvement in this signaling pathway. These data all point to increases in $[Ca^{2+}]_i$ as being central in the regulation of pollen tube growth. The localized nature of $[Ca^{2+}]_i$ transients observed has led us to investigate the possibility that the phosphoinositide (PI) signaling pathway may be involved in the generation of $[Ca^{2+}]_i$ transients.

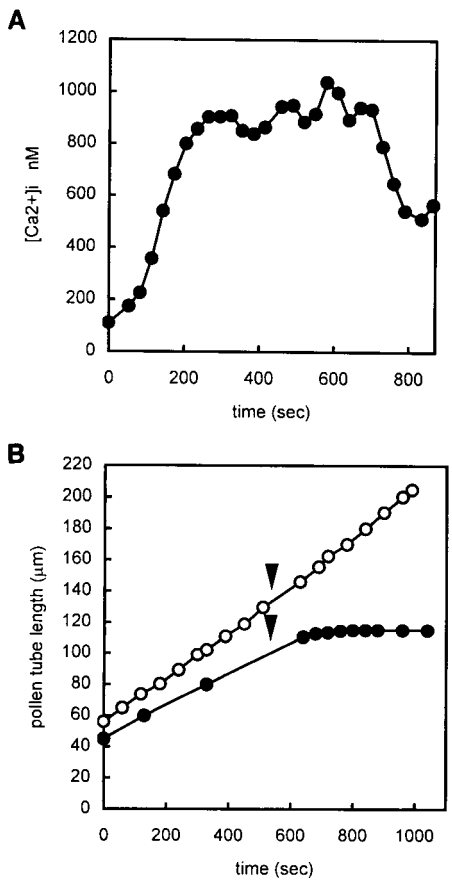

Figure 1. Effect of Release of Caged $Ins(1,4,5)P_3$ on $[Ca^{2+}]_i$ and Pollen Tube Growth.

(A) The dynamics of temporal changes in $[Ca^{2+}]_i$ induced by photolysis of caged $Ins(1,4,5)P_3$ in a growing *P. rhoeas* pollen tube. Growing pollen tubes were microinjected with the calcium-sensitive fluorescent dye CG-1 and caged $Ins(1,4,5)P_3$. After complete recovery of growth, the pollen tube was exposed to 20 sec of UV photolysis at time zero. Changes in $[Ca^{2+}]_i$ were recorded using LSCM for up to 15 min and calibrated, as described in Methods. The data here show a typical temporal response (n = 20).

(B) Effect of release of caged $Ins(1,4,5)P_3$ on *P. rhoeas* pollen tube growth rates. Shown is the growth rate of a typical, representative pollen tube (n = 14) microinjected and loaded with CG-1 and caged $Ins(1,4,5)P_3$ (filled symbols) or uninjected pollen tube (n = 9) (open symbols). Both pollen tubes were exposed to 20 sec of UV photolysis (arrowheads).

A primary mechanism for stimulus-induced $[Ca^{2+}]_i$ elevation in mammalian cells is signal transduction via the PI system (Berridge, 1993). Briefly, agonist–receptor interactions lead to the activation of the enzyme(s) PI-specific phospholipase C, phosphoinositidase C (PIC). This results in the hydrolysis of the membrane phospholipid phosphatidylinositol-(4,5)-bisphosphate ($PtdIns[4,5]P_2$) and production of the two messengers, inositol (1,4,5)-trisphosphate ($Ins[1,4,5]P_3$) and diacylglycerol (DG). Specific receptors for $Ins(1,4,5)P_3$ are localized on specialized Ca^{2+}-containing endomembrane compartments, and when $Ins(1,4,5)P_3$ is bound to its receptors, Ca^{2+} is rapidly released from these intracellular stores. DG, however, remains in the plasma membrane matrix, where it modulates the activity of the enzyme protein kinase C. When the primary signal–receptor complexes dissociate, PIC is inactivated, the cellular levels of $Ins(1,4,5)P_3$ and DG decrease due to hydrolysis, and $[Ca^{2+}]_i$ levels are returned to resting levels due to the removal of Ca^{2+} by several membrane-associated Ca^{2+}-transporting systems.

The possibility that a similar system for the mobilization of intracellular Ca^{2+} exists in higher plants has received considerable attention in recent years. A consensus is emerging that a plant PI system does exist, and functional homologs to the main components of the mammalian PI system have now been identified and characterized (for reviews, see Boss, 1989; Drøbak, 1992; Hetherington and Drøbak, 1992; Coté and Crain, 1993; Drøbak et al., 1996). The ability of $Ins(1,4,5)P_3$ to mobilize Ca^{2+} from intracellular stores in plant cells was first demonstrated in vitro by using various membrane preparations (Drøbak and Ferguson, 1985; Schumaker and Sze, 1987), and subsequent experiments have demonstrated that $Ins(1,4,5)P_3$ can also mobilize intracellular Ca^{2+} in vivo (Gilroy et al., 1990; Shacklock et al., 1992). Furthermore, a putative $Ins(1,4,5)P_3$ receptor has recently been identified and partially purified from plant cells (Brosnan and Sanders, 1990; Biswas et al., 1995). Despite this progress, there is still considerable debate about the physiological roles and precise mode of action of $Ins(1,4,5)P_3$ in plant cells. Here, we have investigated the possible role of $Ins(1,4,5)P_3$ in Ca^{2+} mobilization and regulation of pollen tube growth.

RESULTS

Photolysis of Caged $Ins(1,4,5)P_3$ Induces Release of $[Ca^{2+}]_i$

P. rhoeas pollen tubes growing in vitro were microinjected with caged $Ins(1,4,5)P_3$ and the Ca^{2+} dye Calcium Green-1 (CG-1). $Ins(1,4,5)P_3$ was released by UV photolysis, and the effects on $[Ca^{2+}]_i$ were recorded. The single wavelength dye CG-1 was used for $[Ca^{2+}]_i$ estimation because the Ca^{2+} dyes fura-2 and indo-1, commonly used for $[Ca^{2+}]_i$ imaging, are excited by UV light, making them unsuitable for use with caged probes that are photolyzed at this wavelength. Figure 1A shows a typical

example (n = 20) of the effects of photolysis of caged Ins(1,4,5)P_3 on the changes of $[Ca^{2+}]_i$ in a *P. rhoeas* pollen tube. We estimated (see Methods) that optimized UV photolysis raises the level of intracellular Ins(1,4,5)P_3 by ~0.9 μM.

Despite the fact that the caged Ins(1,4,5)P_3 was released throughout the pollen tube within 10 to 20 sec, unexpectedly there was only a small or undetectable rapid increase in $[Ca^{2+}]_i$. Instead, a characteristic slow increase in $[Ca^{2+}]_i$, which continued for 300 sec and was sustained at high levels for a further 400 sec, was observed. The kinetics shown in Figure 1A suggest that only a small proportion of the Ca^{2+} was directly mobilized by the photolyzed Ins(1,4,5)P_3 and that further mobilization is dependent on other reactions. To investigate the characteristics of this lag in the increase in $[Ca^{2+}]_i$, we imaged the spatial distribution of $[Ca^{2+}]_i$. In Figure 2A, laser scanning confocal microscopy (LSCM) was used to obtain a series of images of a pollen tube every 20 sec after UV photolysis. During the period of slow increase in $[Ca^{2+}]_i$ (Figure 1A), a Ca^{2+} "wave" was initiated, in this case ~150 μm behind the tip. This Ca^{2+} wave traversed the pollen tube, reaching the tip within 100 to 120 sec (see Figure 2A). Additional Ca^{2+} waves were found to continue to move forward after the initial wave reached the pollen tube tip, and $[Ca^{2+}]_i$ continued to increase throughout the tube. After a few minutes, the increases in $[Ca^{2+}]_i$ declined (i.e., it is a transient increase, lasting typically 6 to 10 min, although in some cases the increase was sustained for more than 15 min), as shown in Figure 1A. Although we describe this as a "Ca^{2+} wave," the terms "Ca^{2+} tide" or "Ca^{2+} flood" are perhaps more appropriate because $[Ca^{2+}]_i$ continues to increase for many minutes in regions in which the wave has passed. These increases in Ca^{2+} were not detected in pollen tubes in which no Ins(1,4,5)P_3 was released. Figure 2B shows a typical pollen tube that was microinjected with CG-1 and caged Ins(1,4,5)P_3 but that was not subjected to photolysis.

Correlation between Release of $[Ca^{2+}]_i$ by Caged Ins(1,4,5)P_3 and Pollen Tube Inhibition

Photolysis of caged Ins(1,4,5)P_3 in *P. rhoeas* pollen tubes resulted in detectable rises in Ca^{2+} in 20 of 26 pollen tubes (n = 26). Ca^{2+} waves were detected in 60% of these pollen tubes. A number of pollen tubes that had ceased growing but still exhibited cytoplasmic streaming were also subjected to photolysis of caged Ins(1,4,5)P_3 to determine whether the pollen tubes still retained the ability to respond; an increase in $[Ca^{2+}]_i$ was detectable in every case (n = 4). In 85% of the responding pollen tubes, $[Ca^{2+}]_i$ in the tip region changed within 60 to 180 sec after UV photolysis of Ins(1,4,5)P_3 (n = 20). These increases in $[Ca^{2+}]_i$ correlated well with changes in pollen tube tip growth.

These changes in pollen tube growth were observed as a variety of responses, including inhibition of growth in ~70% of all cases, which we believe occurred as a result of the release of caged Ins(1,4,5)P_3 and subsequent increase in $[Ca^{2+}]_i$. In 45% of the treated pollen tubes, altered tip morphology was observed as tip swelling and flattening. These changes were accompanied by growth cessation, resulting from the arrival of Ca^{2+} at the tip. In other pollen tubes (three of 20), elongation stopped completely when Ca^{2+} in the tip was elevated. The remaining pollen tubes (five of 20) showed a change in tip morphology and a temporary cessation of growth subsequent to Ca^{2+} increases; however, growth then recommenced, often with a change of tip orientation. Pollen tubes whose growth was inhibited usually continued to display active cytoplasmic streaming for up to 30 min and often resulted in callose deposition in the tip region. The inhibition of pollen tube growth triggered by the release of caged Ins(1,4,5)P_3 is illustrated in Figure 1B. It is specific to increases in Ins(1,4,5)P_3 because UV exposure of unloaded pollen tubes (n = 9) had no measurable effect on pollen tube growth (Figure 1B).

It is clear that not all cells respond in the same way, for which there are a number of possible explanations. The microinjection experiments revealed that there is a range of responses found in individual cells; data from a population of cells might give a quite different picture, depending on how synchronized their responses are. We consider that because 77% of the cells respond to the release of caged Ins(1,4,5)P_3 by increasing $[Ca^{2+}]_i$ and 70% have tip growth inhibited and the remainder have tip growth altered in some way, there is a high level of reproducibility for a biological system. A large proportion of pollen tubes with increases in $[Ca^{2+}]_i$ generated a Ca^{2+} wave, but at present it is unclear why not all of them respond in the same way. The lack of response and variation in some cells may be due to differences in amounts of caged molecules injected or released by photolysis. Another possibility is that cells differ in their state of responsiveness. The possible causes for this variation, which are quite usual, are discussed at length by Allan et al. (1994) and McAinsh et al. (1992) with respect to responses to abscisic acid–induced Ca^{2+} increases in *Commelina* guard cells.

Demonstration of Ca^{2+}-Dependent PIC Activity in Pollen

The experiments using caged Ins(1,4,5)P_3 indicated that Ins(1,4,5)P_3 is potentially a physiological regulator of Ca^{2+} fluxes in *P. rhoeas* pollen. This suggests that a functional PI signaling pathway may be operating. An important question is, of course, whether the molecular machinery responsible for the in vivo production of Ins(1,4,5)P_3 exists in these cells. To answer this question, we tested whether a PtdIns(4,5)P_2-specific PIC activity was present in *P. rhoeas* pollen. Figure 3A shows that PIC activity indeed exists in pollen microsomes and is capable of mediating the diesteratic cleavage of PtdIns(4,5)P_2 when this substrate is presented to the enzyme in a micellar conformation. The reaction was found to be near

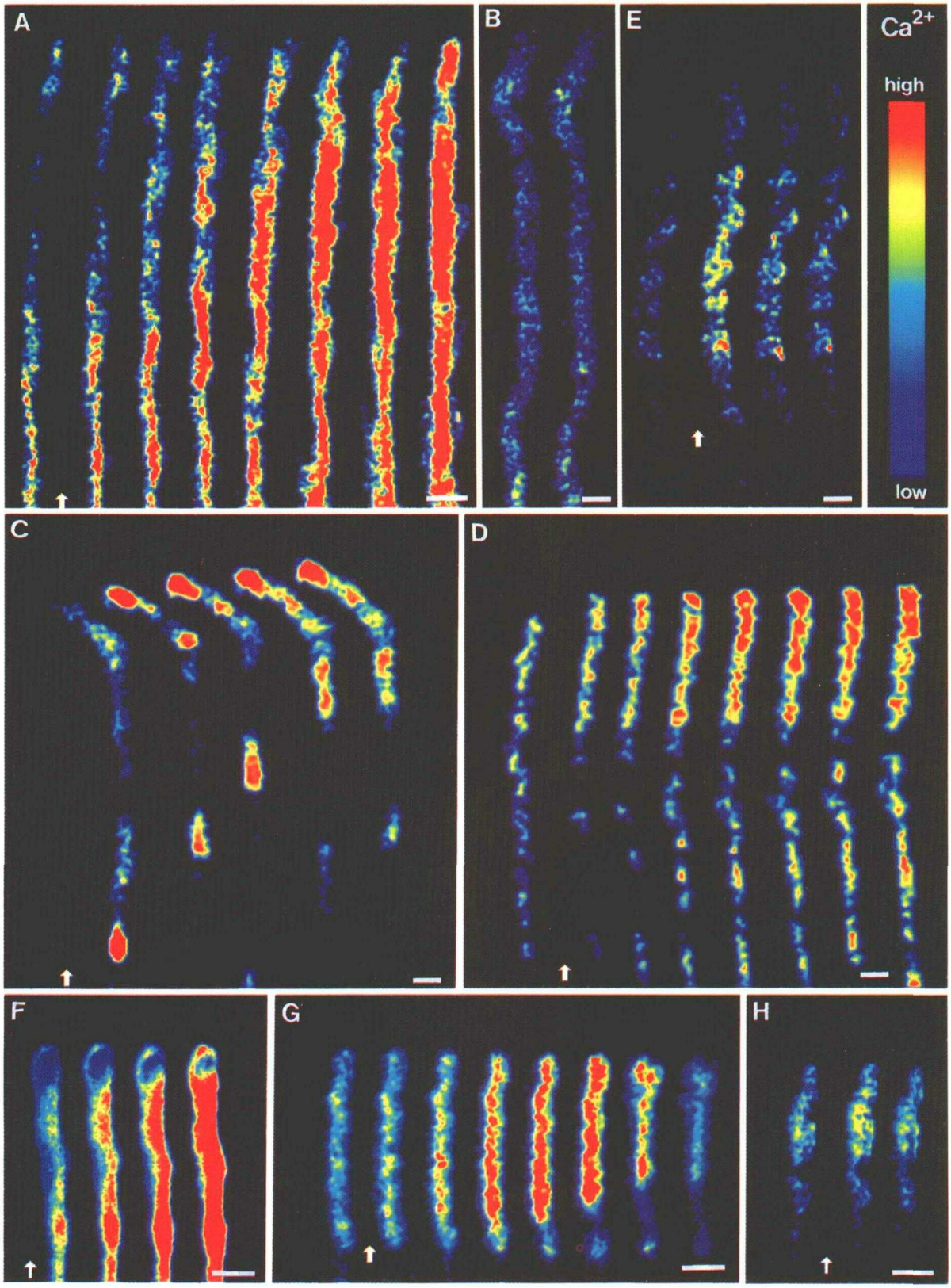

Figure 2. Visualization of Changes in $[Ca^{2+}]_i$ Levels in *P. rhoeas* Pollen Tubes Imaged by LSCM.

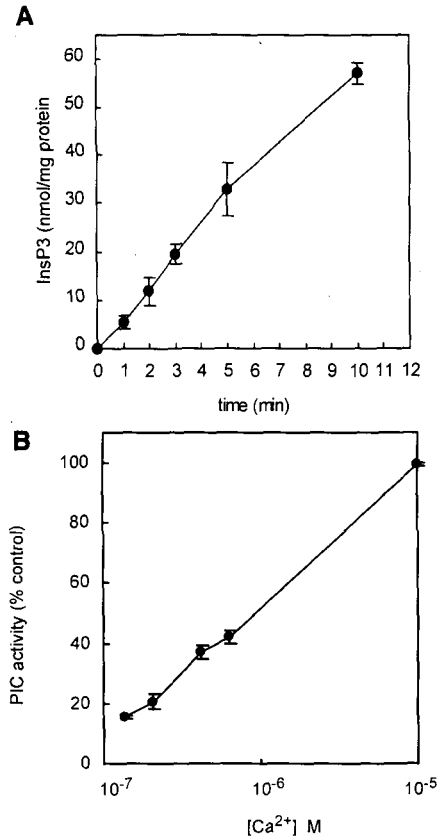

Figure 3. PIC Activity in *P. rhoeas* Pollen Tube Microsomes.

(A) Detection of PIC activity in *P. rhoeas* pollen tube microsomes. Microsomal fractions prepared from pollen tubes were incubated with authenticated ^{3}H-PtdIns(4,5)P_2, and the release of Ins(1,4,5)P_3 was measured. The data are from two independent experiments; vertical bars indicate standard error range.

(B) PIC activity in *P. rhoeas* pollen tube microsomes is Ca^{2+} dependent. PIC activity was measured in the presence of different concentrations of Ca^{2+}. The data are from two independent experiments; vertical bars indicate standard error range.

linear for at least 10 min, as shown in Figure 3. We also investigated the Ca^{2+} dependence of this enzyme activity and found that it was dependent on physiological concentrations of Ca^{2+}, as shown in Figure 3B. This pollen PIC is membrane associated and belongs to the class II group of PIC enzymes (see Drøbak [1992] for details). Its overall activity is comparable to that observed in a wide range of other plant tissues (see Drøbak et al. [1994] for further discussion). These experiments demonstrate that the key enzyme(s) required for rapid Ins(1,4,5)P_3 production is present and active in pollen of *P. rhoeas*.

Mastoparan-Induced Ins(1,4,5)P_3 Production

Mastoparan has been shown to induce rapid increases in the cytosolic levels of Ins(1,4,5)P_3 in both animal and plant cells, although its precise mode of action is currently open to debate (see Discussion). Because *P. rhoeas* pollen has Ca^{2+}-dependent PIC activity, we first investigated whether

Figure 2. (continued).

Ca^{2+} imaging was done by using LSCM of an individual pollen tube for each series. $[Ca^{2+}]_i$ levels in all of these images are represented in pseudocolor, with blue indicating low levels and red indicating high levels of $[Ca^{2+}]_i$, as shown in the color scale. In **(A)** to **(H)**, bars = 10 μm.

(A) Localization of changes in $[Ca^{2+}]_i$ levels in a *P. rhoeas* pollen tube imaged after photolysis of loaded caged Ins(1,4,5)P_3. Growing pollen tubes were microinjected with CG-1 and caged Ins(1,4,5)P_3. After pollen tube growth recommenced, the pollen tubes were exposed to 20 sec of UV photolysis (indicated by the arrow) to release Ins(1,4,5)P_3. LSCM images are of an individual pollen tube at 20-sec intervals up to 120 sec after photolysis.

(B) Levels of $[Ca^{2+}]_i$ in a *P. rhoeas* pollen tube in the absence of UV photolysis of loaded caged Ins(1,4,5)P_3. Control pollen tubes were treated as given in **(A)**, except photolysis was not performed. The images shown here are 80 sec apart.

(C) and **(D)** Effect of treatment with mastoparan on $[Ca^{2+}]_i$ levels in growing *P. rhoeas* pollen tubes. Growing pollen tubes were microinjected with CG-1 and treated by adding 25 μM mastoparan (indicated by the arrows) and imaged. The images in **(C)** and **(D)** show slightly different responses. Images in **(C)** are 20, 60, 80, 100, and 120 sec, respectively, after treatment; in **(D)** they are 10, 20, 30, 40, 50, 60, and 80 sec after treatment.

(E) Changes in $[Ca^{2+}]_i$ imaged in growing *P. rhoeas* pollen tubes containing loaded heparin after photolysis of loaded caged Ins(1,4,5)P_3. Growing pollen tubes were microinjected with CG-1, heparin, and caged Ins(1,4,5)P_3. UV photolysis was performed at the time indicated by the arrow. LSCM images from an individual pollen tube are shown before photolysis and 40 and 160 sec after photolysis.

(F) Changes in $[Ca^{2+}]_i$ levels in growing *P. rhoeas* pollen tubes after treatment with the calcium ionophore Br-A23187. Growing pollen tubes were microinjected with CG-1, and Br-A23187 was added (indicated by the arrow). LSCM images shown are at 30, 60, 110, and 150 sec after the addition of Br-A23187.

(G) Changes in $[Ca^{2+}]_i$ levels in growing *P. rhoeas* pollen tubes after photolysis of loaded nitr-5 (caged Ca^{2+}). Growing pollen tubes were microinjected with CG-1 and nitr-5 and subjected to UV photolysis, as indicated by the arrow. LSCM images shown are at 1, 3, 5, 8, 12, 13, and 14 min after photolysis.

(H) Changes in $[Ca^{2+}]_i$ levels in growing *P. rhoeas* pollen tubes after photolysis of microinjected nitr-5 in the presence of microinjected heparin. Growing pollen tubes were microinjected with CG-1, nitr-5, and heparin. LSCM images shown are at 40, 80, 120, and 160 sec after UV photolysis (indicated by the arrow).

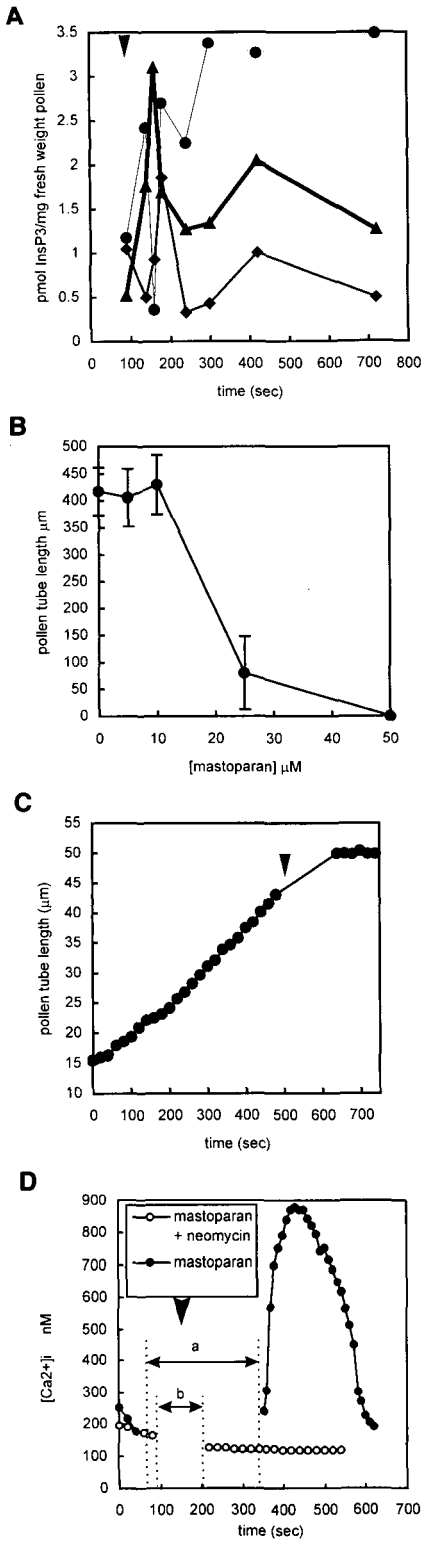

Figure 4. Effect of Addition of Mastoparan to Pollen Tubes of *P. rhoeas*.

(A) Increases in levels of Ins(1,4,5)P_3 ($InsP_3$) in growing *P. rhoeas* pollen tubes treated with mastoparan. Ins(1,4,5)P_3 levels in growing pollen tubes were measured after addition of 25 µM mastoparan (at the arrowhead) over a period of 10 min. The data presented represent three independent experiments, as indicated by the different symbols, and show the temporally similar patterns of Ins(1,4,5)P_3 levels.

(B) *P. rhoeas* pollen tube growth is inhibited by mastoparan. Pollen was germinated and grown in the presence of a range of concentrations of mastoparan. Pollen tube growth was stopped after 2 hr, and pollen tube lengths were measured. The data are from two independent experiments. Mean pollen tube length is shown (n = 20), with standard deviation indicated.

(C) Effect of mastoparan on *P. rhoeas* pollen tube growth rates. Growth of individual pollen tubes was measured before and after addition of 25 µM mastoparan (at the arrowhead). The plot represents a typical response from a single pollen tube.

(D) Changes in $[Ca^{2+}]_i$ in growing *P. rhoeas* pollen tubes treated with mastoparan in the presence and absence of neomycin. Pollen tubes were loaded with CG-1, and changes in $[Ca^{2+}]_i$ were measured in the absence and presence of neomycin. Mastoparan (25 µM) was added at the arrowhead. a and b indicate the resetting period to allow refocusing of the specimen after the addition of mastoparan.

mastoparan could affect Ins(1,4,5)P_3 levels in growing pollen tubes. We then studied whether treatment of pollen tubes with mastoparan causes inhibition of pollen tube growth and investigated whether mastoparan could increase $[Ca^{2+}]_i$ in pollen tubes.

Addition of Mastoparan to Growing Pollen Tubes Increases Ins(1,4,5)P_3 Levels

Figure 4A shows that treatment of pollen tubes with 25 µM mastoparan results in increases in Ins(1,4,5)P_3 levels in a biphasic manner (n = 7). Substantial increases in Ins(1,4,5)P_3 levels (2 to 3 pmol) from a resting level of ~0.5 to 1.0 pmol were detected. Representative examples of individual experiments are shown in Figure 4A to illustrate that although the results are variable, the general profiles observed are similar. There is an initial, very rapid increase in Ins(1,4,5)P_3 produced within 40 to 60 sec. This increase returns to basal levels within 120 sec and is followed by a more sustained increase at ~5 to 6 min, which lasts for several minutes.

Addition of Mastoparan to Pollen Tubes Results in Inhibition of Pollen Tube Growth

Because 25 µM mastoparan appeared to stimulate increases in Ins(1,4,5)P_3 production in pollen, we investigated whether similar concentrations of mastoparan would inhibit pollen tube growth. Figure 4B shows that mastoparan at concentrations of up to 10 µM does not significantly inhibit pollen tube growth, whereas at 25 µM and above, pollen tube growth is strongly inhibited. Treatment of growing pollen tubes with 25 µM mastoparan resulted in inhibition of pollen tube growth within 150 sec of addition (Figure 4C). This similarity in the concentrations of mastoparan required to increase Ins(1,4,5)P_3 and

inhibit growth provided indirect evidence that the increases in Ins(1,4,5)P_3 stimulated by mastoparan could also be responsible for the inhibition of pollen tube growth.

Addition of Mastoparan to Pollen Tubes Results in Increases in $[Ca^{2+}]_i$

Because mastoparan induces an increase in Ins(1,4,5)P_3 levels and inhibits pollen tube growth, we investigated whether this treatment would also increase $[Ca^{2+}]_i$ in pollen tubes. Mastoparan (25 μM) was added to pollen tubes microinjected with CG-1, and $[Ca^{2+}]_i$ was imaged (n = 5). Two examples of different pollen responses are shown in Figures 2C and 2D and illustrate that the initial increase in Ca^{2+} reached the tip very rapidly, traversing the pollen tube within 20 sec. However, there appears to be a continued propagation of increased levels of $[Ca^{2+}]_i$ in the region traversed by the initial increases in Ca^{2+}. In some cases (see Figure 2C), we were able to detect increases in the "nuclear region" before the Ca^{2+} increase reached the tip; in other cases (Figure 2D), increases farther back were detected as $[Ca^{2+}]_i$ continued to increase in the tip region (compare Figures 2C and 2D with Figure 2A). The dynamics of the $[Ca^{2+}]_i$ response to mastoparan are shown in Figure 4D. The increase in $[Ca^{2+}]_i$, which peaked within 250 sec, can be compared with the data in Figure 1A. Although the increase in $[Ca^{2+}]_i$ stimulated by mastoparan could be interpreted to be in the form of a fast-moving $[Ca^{2+}]_i$ wave, it is much faster than that stimulated by photolysis of caged Ins(1,4,5)P_3. Thus, it is unclear whether identical mechanisms are operating in these two events of Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ increases. Nevertheless, these data show that 25 μM mastoparan both stimulates increases in Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ in pollen and completely inhibits pollen tube growth.

Are the Calcium Increases Propagated by Ins(1,4,5)P_3-Induced Ca^{2+} Release?

Because pollen tubes can be stimulated to increase their Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ levels, we investigated the possible mechanism involved in the observed Ca^{2+} increases. One possibility is that Ins(1,4,5)P_3-induced Ca^{2+} release (IICR), which involves activation of PIC by increases in $[Ca^{2+}]_i$, induces the resynthesis of Ins(1,4,5)P_3, thereby mobilizing additional Ca^{2+} from intracellular stores. We used the two inhibitors neomycin and heparin to explore the hypothesis that the increases in Ca^{2+} observed are due to IICR. The advantages and limitations of this approach are evaluated in the Discussion.

Neomycin Inhibits PIC Activity and Pollen Tube Growth

Neomycin has been shown to inhibit PIC activation and IICR in both plant and animal systems. Because neomycin is reported to be readily cell permeable, we tested the effect of a range of concentrations on PIC activity in *P. rhoeas* pollen microsomes; the results are shown in Figure 5A. Neomycin had no detectable effect on PIC activity at 100 mM or below but caused virtually complete inhibition of PIC activity at and above 150 μM. The effect of neomycin on pollen tube growth was also tested. The results are shown in Figure 5B. Although neomycin had a detectable effect on pollen tube growth at concentrations between 0 to 50 μM, pollen tube growth was severely inhibited only when concentrations of 100 μM or greater were used. At 150 μM neomycin, pollen tube growth was almost completely inhibited and pollen tube elongation was inhibited virtually immediately, although cytoplasmic streaming continued for some time. This suggests a possible requirement for PI turnover for normal pollen tube growth.

Heparin Inhibits Pollen Tube Growth

Heparin was used to complement the neomycin experiments. Heparin is a widely used competitive inhibitor of Ins(1,4,5)P_3 receptor binding. Although one problem with its use in intact cells is that it is not cell permeable, growing pollen tubes, unlike plant suspension cells, are known to take up quite large molecules (e.g., 4 to 10 kD) within 15 to 30 min (O'Driscoll et al., 1993), so the effect of low molecular mass heparin (3 kD) on *P. rhoeas* pollen tube growth was tested. Figure 5C shows the results of these experiments. Low molecular mass heparin at 1 mg mL^{-1} external concentration had no or very little effect on pollen tube growth, whereas 5 to 10 mg mL^{-1} resulted in virtually complete inhibition of pollen tube growth. High molecular mass heparin (18 kD) applied externally had no effect on pollen tube growth (data not shown). The fact that low molecular mass heparin affected pollen tube growth suggests the possible requirement for Ins(1,4,5)P_3 binding to its receptor for normal pollen tube growth. However, although one can deduce that low molecular mass heparin does enter the pollen tubes, it is difficult to estimate the intracellular concentration required for inhibition. For this reason, heparin was microinjected into pollen tubes, because it provides a better (although still approximate) estimate of intracellular concentrations. Microinjection of heparin (final concentration of ~100 μg mL^{-1}) resulted in immediate inhibition of pollen tube growth (data not shown).

Heparin and Neomycin Block Ca^{2+} Release Induced by Photolysis of Caged Ins(1,4,5)P_3

To examine further the effect of heparin on $[Ca^{2+}]_i$ release in *P. rhoeas* pollen, growing pollen tubes were microinjected with CG-1, heparin, and caged Ins(1,4,5)P_3 and photolyzed to release Ins(1,4,5)P_3 (n = 10). In the presence of heparin, increases in $[Ca^{2+}]_i$ were blocked either completely or partially. The imaging data in Figure 2E show the spatial localization characteristic of the incomplete inhibition of the

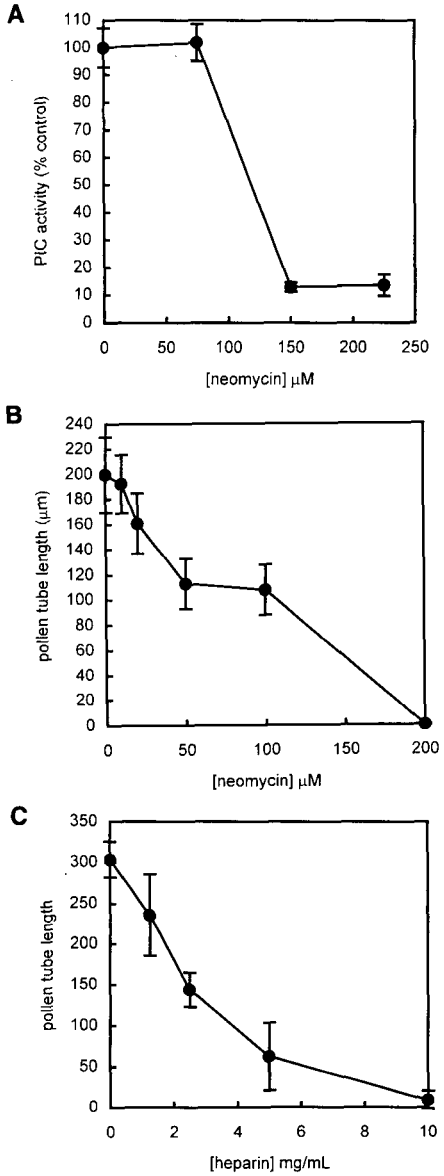

Figure 5. Effect of Neomycin and Heparin on *P. rhoeas* PIC Activity and Pollen Tube Growth.

(A) PIC activity in *P. rhoeas* pollen tube microsomes is inhibited by neomycin. PIC activity was measured, as given in Figure 3, in the presence of different concentrations of neomycin, with standard deviations indicated.

(B) *P. rhoeas* pollen tube growth is inhibited by neomycin. Pollen was germinated and grown in the presence of a range of concentrations of neomycin. Pollen tube growth was stopped after 2 hr, and pollen tube lengths were measured. Mean pollen tube length is shown (n = 30), with standard deviation indicated.

(C) *P. rhoeas* pollen tube growth is inhibited by heparin. Pollen was germinated and grown in the presence of a range of concentrations of low molecular mass heparin. Pollen tube growth was stopped after 2 hr, and pollen tube lengths were measured. The data are from six independent experiments. Mean pollen tube length is shown (n = 60), with standard deviation indicated.

$[Ca^{2+}]_i$ increases. Typically, any increases in $[Ca^{2+}]_i$ in the presence of heparin were highly localized and never reached the tip. Increases in $[Ca^{2+}]_i$ were not large and were short-lived, returning to resting levels within ~100 sec, whereas in the absence of heparin, they continued to increase for more than 600 sec (see Figure 1A). Figure 6 shows the virtually complete block of $[Ca^{2+}]_i$ release by heparin. Pollen tubes microinjected with CG-1 and caged Ins(1,4,5)P_3 were also treated with neomycin before the release of caged Ins(1,4,5)P_3. These results are also shown in Figure 6. After photolysis of caged Ins(1,4,5)P_3, no $[Ca^{2+}]_i$ increases could be detected by imaging (n = 5), indicating that neomycin completely inhibits the increases in $[Ca^{2+}]_i$. Because both heparin and neomycin strongly impair the gradual, slow $[Ca^{2+}]_i$ increases or waves normally stimulated by caged Ins(1,4,5)P_3, our data indicate the involvement of an IICR mechanism in the generation of the increases in $[Ca^{2+}]_i$.

Neomycin Blocks Ca^{2+} Release Induced by Mastoparan

We further tested the hypothesis that the $[Ca^{2+}]_i$ increases in mastoparan-treated pollen tubes were induced by Ins(1,4,5)P_3. Pollen tubes microinjected with CG-1 were pretreated with 150 μM neomycin before mastoparan treatment (n = 4). Figure 4D shows that in contrast to pollen tubes that had received no pretreatment before addition of mastoparan, those pretreated with neomycin showed no detectable changes in $[Ca^{2+}]_i$. These data directly implicate PIC activity in generat-

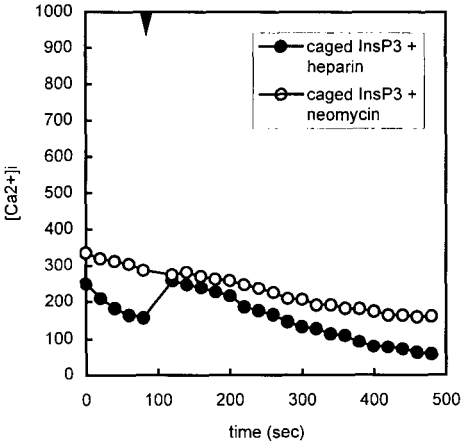

Figure 6. Effect of Loaded Heparin and Pretreatment with Neomycin on Changes in $[Ca^{2+}]_i$ Induced in Growing *P. rhoeas* Pollen Tubes after UV Photolysis of Loaded Caged Ins(1,4,5)P_3.

Growing pollen tubes were loaded with CG-1, caged Ins(1,4,5)P_3 ($InsP_3$), and heparin (filled circles) or with CG-1 and caged Ins(1,4,5)P_3 and pretreated with neomycin for several minutes (open circles). Changes in $[Ca^{2+}]_i$ in a representative individual pollen tube show a typical temporal response after photolytic release of caged Ins(1,4,5)P_3 (at the arrowhead).

ing the mastoparan-induced Ca^{2+} increases, thereby providing good evidence that the detected increases in Ca^{2+} are Ins(1,4,5)P_3 induced.

An Increase in $[Ca^{2+}]_i$ Can Induce Slow Ca^{2+} Increases

We also tested whether the slow increases in Ca^{2+} in pollen tubes could be initiated by increases in $[Ca^{2+}]_i$ itself. Because Gilkey et al. (1978) previously observed that Ca^{2+} waves could be induced in the medaka fish egg by using a Ca^{2+} ionophore, we tried this treatment in pollen tubes. Pollen tubes microinjected with CG-1 were treated with Br-A23187 (n = 4) and imaged. Figure 2F shows a slow increase in Ca^{2+}, in the form of a wave moving toward the tip, with a continuing accumulation of $[Ca^{2+}]_i$ thereafter. These data suggest that Ca^{2+} influx can also stimulate slow increases in Ca^{2+}, which take the form of a wave. Although A23187 can equilibrate Ca^{2+} across membranes with some specificity, it also affects membrane permeability. Thus, its effect may be similar to that induced by mastoparan. However, we have previously observed slow increases in $[Ca^{2+}]_i$ as a result of photolysis of nitr-5 (caged Ca^{2+}) (Franklin-Tong et al., 1993), which increases $[Ca^{2+}]_i$ without the involvement of external Ca^{2+}. Figure 2G shows a pollen tube following nitr-5 photolysis, and Figure 7 shows the kinetics of the $[Ca^{2+}]_i$ increase induced by release of caged Ca^{2+}. It can be seen that Ca^{2+} increases over a period of >200 sec, although nitr-5 is released at time zero. An immediate increase in $[Ca^{2+}]_i$ was followed by a slower and more prolonged increase (n = 6), which appears very similar to that shown in Figures 2A, 2C, 2D, and 2F. Thus, an elevation in Ca^{2+} itself, not necessarily involving Ca^{2+} influx, is sufficient to initiate the slow Ca^{2+} increases.

Because the increases in Ca^{2+} originally detected appeared to be propagated by Ins(1,4,5)P_3, we tested whether they could also be initiated by increases in $[Ca^{2+}]_i$ and then propagated by Ins(1,4,5)P_3. Pollen tubes microinjected with CG-1, heparin, and nitr-5 were subjected to photolysis and imaged. Figure 2H illustrates that there was an immediate but very small transient increase in $[Ca^{2+}]_i$. The kinetics and quantitation of this type of response are represented in Figure 7, which shows that the $[Ca^{2+}]_i$ declines almost immediately (n = 4). These data imply that the slow increases in $[Ca^{2+}]_i$ can be initiated by an increase in $[Ca^{2+}]_i$ itself but further support the view that propagation is dependent on Ins(1,4,5)P_3.

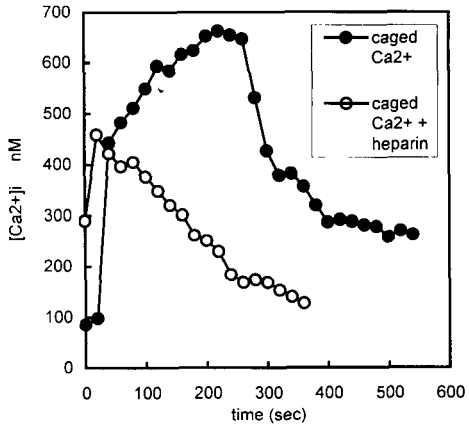

Figure 7. Changes in $[Ca^{2+}]_i$ Levels in Growing *P. rhoeas* Pollen Tubes after Photolysis of Loaded nitr-5 in the Presence and Absence of Loaded Heparin.

Growing pollen tubes were loaded with CG-1 and nitr-5, and UV photolysis was performed at time zero in the presence (open circles) and absence (filled circles) of loaded heparin.

DISCUSSION

A Role for Ins(1,4,5)P_3 in Regulating Intracellular Ca^{2+} in Pollen Tubes

Relatively little has been done to investigate PI metabolism in pollen tubes. Nevertheless, Helsper et al. (1986, 1987) have demonstrated the presence of both phosphoinositides and a PIC activity in pollen. However, they did not establish whether this pollen PIC enzyme(s) had the ability to hydrolyze PtdIns(4,5)P_2. We provide direct evidence for the presence of a Ca^{2+}-dependent PIC, which is capable of hydrolyzing PtdIns(4,5)P_2, leading to the production of Ins(1,4,5)P_3 in pollen. We also demonstrate that increases in Ins(1,4,5)P_3 can be stimulated in growing pollen tubes. Little is known about the precise mode of activation of plant PIC, but it is known that this enzyme is totally dependent on Ca^{2+} in the physiological range when assayed in vitro (see Melin et al., 1992; Drøbak et al., 1994; Hirayama et al., 1995; Huang et al., 1995).

Although our data indicate the potential for rapid synthesis of Ins(1,4,5)P_3 in pollen, Ins(1,4,5)P_3 in many plant tissues can be rapidly metabolized by phosphatases and kinases (Joseph et al., 1989; Drøbak et al., 1991; Drøbak, 1992). The speed with which the initial spike of Ins(1,4,5)P_3 in pollen tubes is degraded suggests that such rapid conversion of Ins(1,4,5)P_3 is likely to occur in pollen tubes. We attempted to establish the presence of polyphosphoinositides in *P. rhoeas* pollen by phosphorus-32 labeling, phospholipid extraction, and separation and analysis by thin-layer chromatography and HPLC. Although we found rapid and substantial radiolabeling of PtdIns(4)P, only extremely low levels of radiolabel could be detected in PtdIns(4,5)P_2. This pattern is also observed in many other plant tissues (Hetherington and Drøbak, 1992). The reason for the very low level of tracer incorporation into PtdIns(4,5)P_2 remains unknown, but this does not preclude very rapid Ins(1,4,5)P_3 production, provided that the turnover rate of PtdIns(4,5)P_2 is upregulated in synchrony with PIC activation (B.K. Drøbak and V. Stavar, unpublished data). Thus, there is scope for sustained increases in $[Ca^{2+}]_i$.

Using Ca^{2+} imaging, we have shown that increases in Ins(1,4,5)P_3 in pollen tubes result in increases in $[Ca^{2+}]_i$. Photolysis of caged Ins(1,4,5)P_3 has been used previously to demonstrate the involvement of Ins(1,4,5)P_3 in the regulation of guard cell aperture (Blatt et al., 1990; Gilroy et al., 1990) and in the control of the volume of etiolated wheat leaf protoplasts (Shacklock et al., 1992). In this study, the levels of Ins(1,4,5)P_3 initiating the Ca^{2+} increases were ~1 μM. This is comparable to Ins(1,4,5)P_3 levels that elicited inward currents in isolated plant vacuoles (Allen and Sanders, 1994) and Ca^{2+} release in smooth muscle fiber cells (Iino and Endo, 1992). Mastoparan increased Ins(1,4,5)P_3 levels in pollen tubes to ~2 to 3 pmol mg^{-1} fresh weight, which is similar to those observed in stimulated carrot cells (Drøbak and Watkins, 1994). These increases in Ins(1,4,5)P_3 were accompanied by increases in $[Ca^{2+}]_i$. The elevation of Ca^{2+} described in this study is therefore likely to be initiated by physiologically relevant concentrations of Ins(1,4,5)P_3.

Both heparin and neomycin potently inhibited the increases in $[Ca^{2+}]_i$, indicating that PIC activity and/or heparin-sensitive Ins(1,4,5)P_3 receptors could be responsible for the observed increases in Ca^{2+}. However, interpretation of data obtained using pharmacological agents, such as mastoparan, neomycin, and heparin, should be approached with caution because there are a number of problems associated with their use. Mastoparan is an amphiphilic peptide that can modulate a number of membrane-associated signaling enzymes, for example, phospholipases A, C, and D in mammalian cells. It is also known to interact with a number of other proteins that (indirectly) could influence PIC activity (see Drøbak and Watkins, 1994; Cho et al., 1995). However, there is definitive evidence that mastoparan at low micromolar concentrations is effective in stimulating PtdIns(4,5)P_2 hydrolysis and causing rapid increases in cytosolic levels of Ins(1,4,5)P_3 in both animal and plant systems (Wallace and Carter, 1989; Legendre et al., 1993; Drøbak and Watkins, 1994; Cho et al., 1995). Mastoparan can also cause PtdIns(4,5)P_2 hydrolysis, which is accompanied by Ca^{2+} influx, although it is unclear to what extent the Ca^{2+} influx is required for the activation of PIC (Choi et al., 1992). Nevertheless, in plant cells, mastoparan remains one of the few well-characterized effective activators of PtdIns(4,5)P_2 hydrolysis (Cho et al., 1995). Therefore, irrespective of its precise mode of action, mastoparan remains a potent tool for the study of events linked to Ins(1,4,5)P_3 production in plant cells.

The two inhibitors neomycin and heparin have been used extensively to investigate involvement of PI cycle metabolites in signaling cascades in many animal cells (e.g., Frank and Fein, 1991). Many naturally occurring polyamines, such as neomycin, interact with PIs (see, for example, Sayers and Michelangeli, 1993). Neomycin has been shown to interact directly with Ins(1,4,5)P_3 and the Ins(1,4,5)P_3 receptor (Prentki et al., 1986; Sayers and Michelangeli, 1993), and there is extensive evidence for the ability of neomycin to inhibit IICR. In plants, neomycin has been shown to inhibit the PIC activity in plasma membranes (Chen and Boss, 1991) and to inhibit Ins(1,4,5)P_3 production within seconds in both soybean suspension culture cells and in intact cells of the alga *Chlamydomonas* (Quarmby et al., 1992; Legendre et al., 1993). There is evidence that neomycin partially inhibits ryanodine binding to mammalian Ca^{2+}-release channels (Wang et al., 1996). This might implicate Ca^{2+}-induced Ca^{2+} release (CICR) involvement because the ryanodine receptor is known to be responsible for CICR. However, Wang et al. (1996) found that the neomycin binding site appears to be distinct from the ryanodine binding site.

Although neomycin is commonly used at fairly high concentrations (1 to 5 mM) in animal cells, lower levels (100 to 300 μM) are effective in plant cells for inhibiting Ins(1,4,5)P_3 accumulation (Legendre et al., 1993). We have shown that comparable levels of neomycin (150 μM) are very effective at inhibiting PIC activity in pollen microsomes and in preventing increases in $[Ca^{2+}]_i$ in pollen tubes stimulated by either mastoparan or Ins(1,4,5)P_3. Cho et al. (1995) have shown that neomycin is antagonistic to the mastoparan analog Mas7 and prevents its uptake in carrot suspension cells. However, because neomycin inhibits not only the effects of mastoparan but also inhibits Ins(1,4,5)P_3-induced Ca^{2+} release in pollen tubes and PIC activity, we have good reason to assume that neomycin is likely to interfere with Ins(1,4,5)P_3 production in vivo.

Heparin is a commonly used Ins(1,4,5)P_3 receptor antagonist that has been employed extensively in the study of IICR. Several studies have established that heparin binds with high affinity to the Ins(1,4,5)P_3 binding site on the Ins(1,4,5)P_3 receptor by acting as a competitive and reversible inhibitor and blocking IICR in both animal and plant cells (Worley et al., 1987; Ghosh et al., 1988; Miyazaki et al., 1992; Brosnan and Sanders, 1993; Biswas et al., 1995). A concern when using heparin in the detailed analysis of the Ins(1,4,5)P_3 receptors in cells with both ryanodine receptors and Ins(1,4,5)P_3 receptors is that heparin also binds to ryanodine receptors and can activate CICR. This can lead to confusing interpretations when attempts are made to distinguish between IICR and CICR (Michelangeli et al., 1995). This does not appear to be a concern in the present study because the increases in $[Ca^{2+}]_i$ are potently blocked by heparin. Although there are suggestions that heparin may also bind Ca^{2+}, some recent careful studies have not supported this hypothesis (Mohri et al., 1995). Heparin has been shown to slow down the propagation of Ca^{2+} waves created by sperm-induced activation in sea urchin eggs by decreasing both the amount and the rate of release of Ca^{2+} from intracellular stores. This inhibition of Ca^{2+} release by heparin has been interpreted in favor of IICR being the primary mechanism underlying the generation of such Ca^{2+} transients (Mohri et al., 1995). We believe that our data can be interpreted in a similar manner.

We estimate the concentration of heparin required to block the Ca^{2+} transients to be ~100 μg mL^{-1} in the pollen tube. This is well within the levels generally used in intact cells. Although Kobayashi et al. (1988) showed that 100 μg mL^{-1}

heparin is effective in blocking the Ins(1,4,5)P_3 receptor in muscle tissue, in other cells very high intracellular concentrations of heparin (1 mg mL^{-1} being usual) are commonly required to block IICR. Mohri et al. (1995) discuss this in some detail and suggest that the interaction of heparin with intracellular proteins present at very high levels in intact cells may explain this effect, because homogenates, which require far less heparin to block IICR, contain only ~1% of total cellular proteins. Thus, although very low levels of heparin are effective in inhibiting IICR in plants (e.g., 5 μg mL^{-1} in beet microsomes), these figures derived from in vitro studies are unlikely to be directly comparable to the levels required in intact plant cells, such as the amount of heparin used in the current study.

A Model for IICR in *P. rhoeas* Pollen Tubes

Having discussed some of the caveats that must be considered in interpreting our data, we propose a model for IICR in pollen tubes, which is illustrated in Figure 8. The observed $[Ca^{2+}]_i$ increases can be initiated by $[Ca^{2+}]_i$ itself, and we cannot entirely rule out the idea that Ca^{2+} influx across the plasma membrane may play some part in this increase. At this stage, we cannot rule out that CICR may occur in addition to or in combination with IICR. However, our data indicate that the slow increases in $[Ca^{2+}]_i$ involved in generating the Ca^{2+} waves observed in pollen tubes are most likely a result of a series of Ins(1,4,5)P_3-generating and Ca^{2+}-mobilizing reactions. A scenario can be envisaged whereby a small initial increase in Ins(1,4,5)P_3 levels leads to a limited and localized release of Ca^{2+} from Ins(1,4,5)P_3-sensitive Ca^{2+} stores. This increase in Ca^{2+} may lead to further activation of other additional Ca^{2+}-dependent PICs, renewed synthesis of Ins(1,4,5)P_3, and additional Ca^{2+} release (see Figure 8). A similar cascade or "domino effect" could be initiated by an increase in Ca^{2+} (from either internal or external sources), which would stimulate PIC activity, increase the rate of Ins(1,4,5)P_3 formation, and lead to Ca^{2+} release.

The proposed model raises the question of whether a threshold concentration of $[Ca^{2+}]_i$ and Ins(1,4,5)P_3 needs to be exceeded before the cascade becomes operative. Some of the parameters important in regulating IICR include interactions among $[Ca^{2+}]_i$, $[Ca^{2+}]_i$-buffering capacity, and the kinetics of Ins(1,4,5)P_3 production/hydrolysis (Alexandre and Lassalles, 1992). Our data suggest that relatively low levels of $[Ca^{2+}]_i$ (~500 nM) are required to trigger the Ca^{2+} wave; this is very similar to the concentrations required for prolonged $[Ca^{2+}]_i$ elevation and closure in guard cells, for example (Gilroy et al., 1990). Increases in Ca^{2+} have been studied in a number of plant cell types, and those observed in the guard cell are closest in behavior to those described in our study, as elevations of $[Ca^{2+}]_i$ levels continue to increase for 10 to 15 min. In contrast, wheat leaf protoplasts and *Agapanthus* pollen tubes appear not to use similar cascades (Shacklock et al., 1992; Malhó et al., 1994).

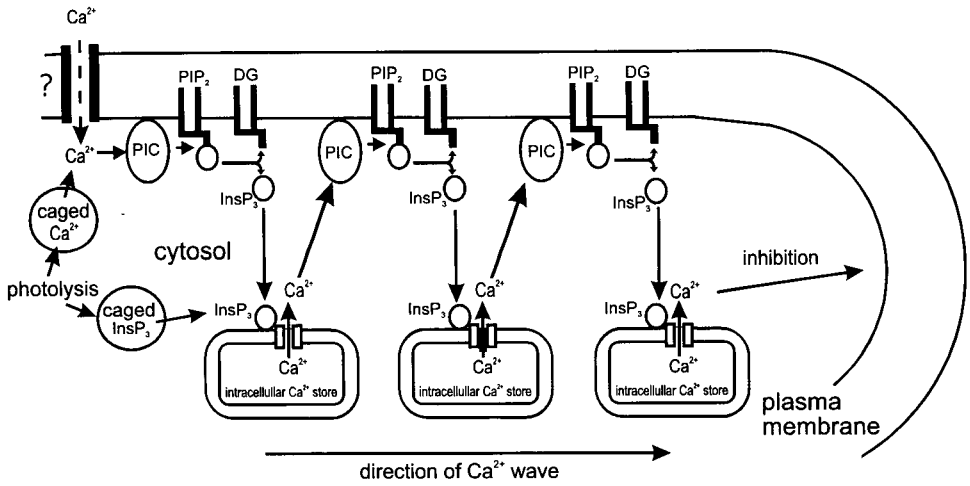

Figure 8. Model for the Propagation of the Ca^{2+} Wave in *P. rhoeas* Pollen Tubes.

The Ca^{2+} wave may be initiated by a small increase in either Ca^{2+} and/or Ins(1,4,5)P_3 ($InsP_3$). Increases in $[Ca^{2+}]_i$ will activate Ca^{2+}-sensitive PICs, which will then hydrolyze the membrane lipid PtdIns(4,5)P_2 (PIP_2) to DG and Ins(1,4,5)P_3. Increased cytosolic levels of Ins(1,4,5)P_3 will stimulate the release of additional Ca^{2+} from Ins(1,4,5)P_3-sensitive intracellular stores. Cytoplasmic streaming in the forward direction occurs in the cytoplasm immediately adjacent to the plasma membrane and may contribute to the wave direction. The continued Ca^{2+} increase may occur as a result of a cascade or "domino" effect of activation of PICs, resulting in continued generation of Ca^{2+} waves from the initiation site. When the wave reaches the pollen tube tip, inhibition of growth results, presumably due to the disruption of the Ca^{2+} gradient at the tip.

A Ca^{2+} Wave Can Be Generated in Growing *P. rhoeas* Pollen Tubes

The spatial and temporal pattern of the IICR observed was unexpected. We believe our data establish that Ca^{2+} waves can be generated in growing *P. rhoeas* pollen tubes. Once these waves reach the tip, the concomitant inhibition of growth is likely to result from the disruption of the tip-based Ca^{2+} gradient, which is thought to be essential for growth. Calcium waves have not been reported previously in plant cells, although Felle (1988) and more recently McAinsh et al. (1995) have described oscillations of $[Ca^{2+}]_i$ in coleoptiles and guard cells. The distinction we make between a wave and an oscillation is that a wave has some spatial alteration, whereas an oscillation implies little or no movement. Ca^{2+} waves have been described extensively in the animal literature, especially in egg cells, where IICR leads to increases in Ca^{2+} which propagate from the point of initial fertilization across the cell (see Jaffe [1993] and Jouaville et al. [1995] for a recent discussion). The data of McAinsh et al. (1995) suggest that Ca^{2+} oscillations in guard cells can result from Ca^{2+} mobilized from both internal and external calcium stores. Our data suggest that this also could be the case in pollen tubes but point to a more direct role of $Ins(1,4,5)P_3$ in generating and maintaining the Ca^{2+} wave. We have already discussed the possible role of IICR, but the details of how this phenomenon may lead to the generation of the Ca^{2+} wave are unclear at present.

It could be argued that the Ca^{2+} wave could be due to movement of $[Ca^{2+}]_i$ within the pollen tube. However, the main movement of cytoplasmic streaming is well documented as an "inverse fountain" type (Iwanami, 1956; Heslop-Harrison and Heslop-Harrison, 1990), whereby the main movement of organelles and cytoplasm is in the opposite direction of that of the Ca^{2+} wave. If $[Ca^{2+}]_i$ were carried by streaming, it would be expected to occur in the very narrow region adjacent to the plasma membrane, where cytoplasm is carried back to the tip. Nevertheless, cytoplasmic streaming in this direction could contribute to the Ca^{2+} wave by locally increasing $[Ca^{2+}]_i$ in the vicinity of the PICs, thereby generating the domino effect.

On the Nature of the $Ins(1,4,5)P_3$-Mobilizable Ca^{2+} Stores

The pollen tube is highly polarized and is characterized by distinct zones comprising a vesicle-rich apical region, a zone rich in endoplasmic reticulum and mitochondria, and a region containing all organelle types, including the nuclei ~100 to 150 μm from the tip. Toward the very back of the tube is a highly vacuolated region (Steer and Steer, 1989). The Ca^{2+} increases induced by caged $Ins(1,4,5)P_3$ were all initiated at least 100 μm from the tip, although they conceivably could originate farther back. Information on the localization and concentration of the $Ins(1,4,5)P_3$-mobilizable Ca^{2+} stores is crucial for understanding how this wave is created and carried.

Currently, the vacuole is considered to be the major $Ins(1,4,5)P_3$-sensitive Ca^{2+} store in higher plants (Schumaker and Sze, 1987; Ranjeva et al., 1988; Canut et al., 1993; Allen and Sanders, 1994). It appears likely that IICR in pollen tubes may be initiated by a vacuolar compartment. Thus, if $Ins(1,4,5)P_3$ increases were distributed evenly in the cell (as assumed for caged $Ins[1,4,5]P_3$ release) and if the $Ins(1,4,5)P_3$ receptors were localized and concentrated in the vacuole, the IICR wave would be expected to be initiated in this region. However, because pollen tubes have no easily identifiable major vacuole in the region in which the Ca^{2+} wave is observed, the possibility of other, additional $Ins(1,4,5)P_3$-sensitive Ca^{2+} stores cannot be ignored. Some doubt has already been cast on the vacuole as the only in vivo target for IICR in plants (Chasan and Schroeder, 1992), and Hepler et al. (1990) have suggested that the cortical endoplasmic reticulum could be a target for $Ins(1,4,5)P_3$-induced Ca^{2+} release in plants. The possibility that $Ins(1,4,5)P_3$ receptors also could be located in the plasma membrane, as reported in certain mammalian cells (Kuno and Gardner, 1987; Krauss-Friedmann, 1994), should not be overlooked, although at present there is nothing to suggest that such a phenomenon occurs in plant cells.

A Role for the PI Signaling Pathway in Mediating Pollen Tube Growth

In summary, we present data strongly suggesting that a functional PI signaling pathway is required for normal pollen tube growth. However, not only do increases in $Ins(1,4,5)P_3$ and the consequent elevation of $[Ca^{2+}]_i$ inhibit pollen tube growth, but inhibition of PI turnover and inhibition of $Ins(1,4,5)P_3$ binding to its receptor also lead to an inhibition of pollen tube growth. These findings suggest that a two-tier level of control involving PI signaling modulates pollen tube growth. We have preliminary data suggesting that small $Ins(1,4,5)P_3$-induced $[Ca^{2+}]_i$ increases could play a role in the reorientation of pollen tube tip growth and that the self-incompatibility response in *P. rhoeas* pollen may also generate a Ca^{2+} wave moving toward the tip (V.E. Franklin-Tong, unpublished data). The idea that Ca^{2+} plays a central role in the control of pollen tube growth is not new (see Introduction), and previously we have established that pollen of *P. rhoeas* uses a signal transduction pathway involving $[Ca^{2+}]_i$ in mediating pollen tube inhibition in the self-incompatibility response (Franklin-Tong et al., 1993, 1995). The control of pollen tube tip growth is clearly a complex process and warrants further study. We envisage that signals controlled by Ca^{2+} influx, IICR, and perhaps CICR are capable of giving complex control of $[Ca^{2+}]_i$ levels, with the possibility of multilevel feedback mechanisms. This hypothesis should provide an exciting starting point for further detailed investigation of PI-mediated Ca^{2+} signaling mechanisms in pollen tubes.

METHODS

Plant Material

Self-incompatibility in *Papaver rhoeas* is controlled by a single locus (*S*-), which is multiallelic. Plants (*P. rhoeas* var Shirley) segregating for known incompatibility genotypes (S_1S_3 and S_4S_5) were used for these experiments (see Franklin-Tong et al., 1988). Pollen was stored at −20°C, over silica gel, until required.

In Vitro System for Growing Pollen for Microinjection

Pollen was grown using an in vitro system adapted for microinjection of pollen tubes, as described by Franklin-Tong et al. (1993). Prehydrated pollen was sown in vitro on thin layers of germination medium solidified with agarose on coverslips (22 × 50 mm) and allowed to germinate and grow for ~1 hr.

Testing the Effects of Inhibitors on Pollen Tube Growth

When sensitivity of pollen to inhibitors was tested, dilution series of the relevant inhibitors (neomycin, heparin, and mastoparan) were made in liquid germination medium comprising 7.5% sucrose, 5% polyethylene glycol 6000, 0.01% KNO_3, 0.01% H_3BO_3, 0.01% $Mg(NO_3)_2 \cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$. Pollen was grown using an in vitro system in which prehydrated pollen was sown in liquid germination medium on multiwell slides (ICN Biomedicals, Inc., Aurora, OH) for 2 hr at 20°C and fixed by adding 2% glutaraldehyde, and pollen tube lengths were measured. Between two and six replicate treatments were used, with measurements made on random samples of between 10 and 30 pollen tubes per treatment. Mean pollen tube length and standard deviation were calculated. In some experiments, inhibitors were added to growing pollen tubes, and growth rate was measured before and after addition, using the CoMOS imaging software package (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, UK).

Microinjection and Loading of Pollen Tubes with Calcium Green-1

The fluorescent dye Calcium Green-1 (CG-1) (Molecular Probes Inc., Eugene, OR), which has high affinity and selectivity for free Ca^{2+} and undergoes changes in the level of fluorescence upon binding to Ca^{2+}, was used to visualize changes in $[Ca^{2+}]_i$ induced in individual living pollen tubes by using laser scanning confocal microscopy (LSCM). Pollen tubes grown in vitro were ionophoretically microinjected with 100 μM CG-1, as described by Franklin-Tong et al. (1993, 1995). Dye was delivered to the pollen tube by ionophoresis (0.1 to 0.2 nA for 1 to 2 sec). Pollen tubes that rapidly recovered from microinjection and that continued to grow were selected for experimentation.

Ca^{2+} Imaging

Pollen tube cytosolic free calcium ($[Ca^{2+}]_i$) was analyzed by using a series of images captured by laser scanning microscopy, as described by Franklin-Tong et al. (1993, 1995). A Bio-Rad MRC-600 system was used to excite dye-loaded pollen tubes with a 25-mW argon-ion laser at 488 nm and to detect CG-1 fluorescence at 525 nm (±40 nm half-band width). Pollen tubes microinjected with CG-1 were monitored before and after various treatments, including the release of caged inositol (1,4,5)-trisphosphate (Ins[1,4,5]P_3) and caged Ca^{2+} and treatments with heparin, neomycin, mastoparan, and the ionophore Br-A23187 (see below for details of treatments). A Nikon dry ×20 Plan Apo (NA 0.75) objective (Micro Instruments Ltd., Witney, Oxon, UK) was used in conjunction with a zoom factor of ×3 to obtain confocal or near confocal images of growing pollen tubes. Images were obtained and processed using the CoMOs imaging software package (Bio-Rad). Images were archived on a Panasonic 940 WORM optical disc drive (Panasonic Industrial UK, Slough, Berks, UK), and hard copies were printed on a Mitsubishi CP100 video copy processor (Micro Instruments Ltd., Witney, Oxon, UK).

Calibration of Ca^{2+} Levels

Changes in $[Ca^{2+}]_i$ levels were analyzed by obtaining the pixel values for the first 100 μm of the pollen tubes imaged over the experimental time period. $[Ca^{2+}]_i$ was calibrated using a calcium calibration kit (Molecular Probes) to assign pixel intensity to approximate $[Ca^{2+}]_i$ concentration (see Allan et al., 1994). These measurements should be viewed with the usual caveats on absolute $[Ca^{2+}]_i$ values associated with single wavelength calibration. Calibration with CG-1 is difficult in pollen tubes because dye is rapidly sequestered in the vacuole, resulting in a decreasing signal during the period of experimentation; compensation for this is virtually impossible because the responsiveness of the dye is dependent on dye concentration. However, this implies that the calibration of the rises in $[Ca^{2+}]_i$ is most likely greater than estimated, because any increases detected later in a time series would be larger than those plotted. Images visualizing the spatial distribution of changes are presented as pseudocolored images in which red/yellow indicate high levels of $[Ca^{2+}]_i$, ranging through to blue for low $[Ca^{2+}]_i$ levels.

Microinjection and Photolysis of Caged Ins(1,4,5)P_3 and Caged Ca^{2+}

Pollen tubes for the caged Ins(1,4,5)P_3 experiments were microinjected with 100 μM CG-1 together with 50 to 100 μM D-*myo*-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester (caged Ins[1,4,5]P_3), trisodium salt (Calbiochem Novabiochem (UK) Ltd., Nottingham, UK). Pollen tubes for the caged Ca^{2+} experiments were microinjected with 100 μM nitr-5 (caged Ca^{2+}) (Calbiochem Novabiochem (UK) Ltd.). The caged probes were photoactivated by exposure to 360 nm of UV light through a Plan Apo ×20 objective (NA 0.75), using the Nikon epifluorescence 100-W Hg lamp TMD-EF attachment and a D360/40 exciter filter in a dichroic filter block (Micro Instruments Ltd.).

Calibration of Amount of Caged Ins(1,4,5)P_3 Released in Pollen Tubes

We estimated the amount of Ins(1,4,5)P_3 released by UV photolysis. Small droplets of fluorescein bis(5-carboxymethoxy-2-nitrobenzyl)ether dipotassium salt (caged fluorescein) (Molecular Probes Inc.) were exposed to increasing periods of UV light (1, 2, 5, 10, 15, 20, 30, and 40

sec), and fluorescence was measured. A dilution series of free fluorescein (Sigma) was used to generate a standard curve for fluorescence of free fluorescein, enabling us to construct a photolysis curve. Under the conditions we used, photolysis of between 5 and 20 sec released ~80% of the fluorescein. We then assumed that the lability of caged Ins(1,4,5)P_3 to UV photolysis is similar to that of caged fluorescein. To estimate the concentration of caged Ins(1,4,5)P_3 inside a loaded pollen tube, we measured the fluorescence of CG-1 in the pollen tube. Because CG-1 is negatively charged like caged Ins(1,4,5)P_3 in free-flow electrophoresis during ionophoresis, equivalent proportions are likely to enter the pollen tube. Assuming that the pollen tube is a cylinder with a diameter of between 8 and 10 μm, the average concentration of loaded CG-1 was estimated at 1.0 ± 0.1 M. We estimated that under the photolysis conditions used, Ins(1,4,5)P_3 would increase by 0.9 to 1.0 μM.

Heparin Treatment of Microinjected Pollen Tubes

Pollen tubes were microinjected with 100 μM CG-1 and either 50 to 100 μM caged Ins(1,4,5)P_3 or nitr-5 (caged Ca^{2+}) (Calbiochem Novabiochem), together with 10 mg mL^{-1} heparin (Sigma), which should give an intracellular concentration of ~100 μg mL^{-1}. Photoactivation of caged Ins(1,4,5)P_3 was as described in the previous section. Pollen tubes did not continue growing after microinjection of heparin. However, a clear effect of heparin on the Ca^{2+} levels lends confidence that the pollen tube is capable of responding to signals.

Treatment of Microinjected Pollen Tubes with Neomycin

Pollen tubes microinjected with 100 μM CG-1 and caged Ins(1,4,5)P_3 (see above) were allowed to grow for 5 to 10 min and then were pretreated with 150 μmol neomycin (Sigma) before release of caged Ins(1,4,5)P_3. Images were captured both before and after photolysis. Although pollen tube growth was inhibited by neomycin, normal cytoplasmic streaming continued, indicating that the pollen was still metabolically active.

Treatment of Microinjected Pollen Tubes with Mastoparan and Br-A23187

Pollen tubes microinjected with 100 μM CG-1 (as above) were allowed to grow for 5 to 10 min, and then a 1-μL droplet of 25 μM mastoparan (Sigma) or 10 μM Br-A23187 (Molecular Probes Inc.) was added to the pollen tube. The microscope was then refocused, and imaging of the response commenced. Because of this necessity, there is a period of 1 to 2 min after addition of the mastoparan in which observations could not be made, so rapid changes in Ca^{2+} could have been missed.

Phosphoinositide-Specific Phospholipase C Activity Assays

Assay of phosphoinositide (PI)-specific phospholipase C (PIC) activity was essentially as described by Drøbak et al. (1994). Microsomal vesicles isolated from pollen (15 μg of protein per sample) were incubated at 26°C in 50 mM Tris-malate, pH 6.0, 10 μM $CaCl_2$, and a sonicated micellar suspension of 142 μM phosphatidylinositol-(4,5)-bisphosphate (PtdIns[4,5]P_2) spiked with authentic ^{3}H-PtdIns(4,5)P_2 (Amersham; specific activity of 325.6 GBq mmol). After various incubation times, aliquots were subsampled and the reactions were terminated by 1 mL of chloroform–methanol (2:1 [v/v]). Assay tubes were placed on ice for 5 min, and 0.25 mL of 0.6 N HCl was added to facilitate separation of phases. Tubes were vortexed and centrifuged at 14,000*g* for 2 min. The top phase (400 μL) was removed from each tube, and radioactivity in the H_2O-soluble components was determined by liquid scintillation spectrometry (model 1410; Wallac, Gaithersburg, MD) after the addition of scintillation fluid (Hionic-Fluor; Hewlett-Packard, Beds, UK). All data were corrected for background radiation. The Ca^{2+} dependency of the pollen PIC was tested exactly as described by Drøbak et al. (1994). The effect of neomycin on PIC activity was also tested as above, with the addition of neomycin at 0, 75, 150, and 225 μM in the assay buffer. The PIC activity was plotted as a percentage of the control PIC data (=100%).

Mastoparan Stimulation of Pollen Tubes and Extraction of Ins(1,4,5)P_3

Pollen was prehydrated on a moist chamber and grown in vitro in liquid germination medium (see above) for ~30 min. Three 100-μL samples for controls were taken at 30-sec intervals before the addition of mastoparan (24 mM) (Sigma), after which additional 100-μL samples were taken at the appropriate time intervals over a 10-min period. Samples were quenched with 100 μL of ice-cold $HClO_4$ (10% [w/v]), left on ice for 15 min, and centrifuged (12,000*g*) for 7 min; 100 μL of the supernatant was removed and neutralized with 1.5 M KOH, 60 mM Hepes. Precipitated $KClO_4$ was removed by centrifugation (12,000*g* for 7 min), and the Ins(1,4,5)P_3 concentration in the supernatant was determined using the receptor binding assay (see Drøbak and Watkins [1994] for further details).

Measurement of Ins(1,4,5)P_3 Accumulation

Ins(1,4,5)P_3 binding protein extracts from bovine adrenal glands were prepared according to the protocol of Palmer and Wakelam (1990) and were used for Ins(1,4,5)P_3 determinations, which followed the protocol as given in the commercially available ^{3}H-Ins(1,4,5)P_3 assay kit (model TRK1000; Amersham). Standard curves were constructed using 2-^{3}H-Ins(1,4,5)P_3 (Amersham) as tracer.

ACKNOWLEDGMENTS

V.E.F.-T. wishes to thank Chris Franklin for his continued support and encouragement. We are indebted to Bob Edwardson for kindly providing us with fresh adrenal glands from which the Ins(1,4,5)P_3 binding protein was extracted and to Mike Finney for guiding us through the procedure. Thanks are due to Antony Jones and Sue Clarke for technical assistance and to John Martin and gardening staff for expert horticultural services. V.E.F.-T. is funded by a Biotechnology and Biological Sciences Research Council (BBSRC) Advanced Fellowship. Work in the laboratory of B.K.D. was supported by the BBSRC Intracellular Signaling Initiative, and A.J.T. is funded by BBSRC.

Received March 22, 1996; accepted June 10, 1996.

REFERENCES

Alexandre, J., and Lassalles, J.P. (1992). Intracellular Ca^{2+} release by inositol (1,4,5)-trisphosphate in plants and effect of buffers on Ca^{2+} diffusion. Philos. Trans. R. Soc. Lond. Ser. B **338,** 53–61.

Allan, A.C., Fricker, M.D., Ward, J.L., Beale, M.H., and Trewavas, A.J. (1994). Two transduction pathways mediate rapid effects of abscisic acid in *Commelina* guard cells. Plant Cell **6,** 1319–1328.

Allen, G.J., and Sanders, D. (1994). Two voltage-gated, calcium release channels coreside in the vacuolar membrane of broad bean guard cells. Plant Cell **6,** 685–694.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signaling. Nature **361,** 315–325.

Biswas, S., Dalal, B., Sen, M., and Biswas, B.B. (1995). Receptor for *myo*-inositol trisphosphate from the microsomal fraction of *Vigna radiata*. Biochem. J. **306,** 631–636.

Blatt, M.R., Thiel, G., and Trentham, D.R. (1990). Reversible inactivation of K^+ channels of *Vicia* stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. Nature **346,** 766–769.

Boss, W.F. (1989). Phosphoinositide metabolism: Its relation to signal transduction in plants. In Second Messengers in Plant Growth and Development, W.F. Boss and D.J. Morre, eds (New York: Allan R. Liss), pp. 29–56.

Brewbaker, J.L., and Kwack, B.H. (1963). The essential role of calcium ions in pollen germination and pollen tube growth. Am. J. Bot. **50,** 859–863.

Brosnan, J.M., and Sanders, D. (1990). Inositol trisphosphate–mediated Ca^{2+} release in beet microsomes is inhibited by heparin. FEBS Lett. **260,** 70–72.

Brosnan, J.M., and Sanders, D. (1993). Identification and characterization of high-affinity binding sites for inositol trisphosphate in red beet. Plant Cell **5,** 931–940.

Bush, D.S. (1995). Calcium regulation in plant cells and its role in signaling. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 95–122.

Canut, H., Carrasco, A., Rossignol, M., and Ranjeva, R. (1993). Is vacuole the richest store of IP3-mobilizable calcium in plant cells? Plant Sci. **90,** 135–143.

Chasan, R., and Schroeder, J.I. (1992). Excitation in plant membrane biology. Plant Cell **4,** 1180–1188.

Chen, Q.Y., and Boss, W.F. (1991). Neomycin inhibits the phosphotidylinositol monophosphate and phosphatidylinositol bisphosphate stimulation of plasma-membrane ATPase activity. Plant Physiol. **96,** 340–343.

Cho, M.H., Tan, Z., Erneux, C., Shears, S.B., and Boss, W.F. (1995). The effects of mastoparan on the carrot cell plasma-membrane polyphosphoinositide phospholipase C. Plant Physiol. **107,** 845–856.

Choi, O.H., Padgett, W.L., and Daly, J.W. (1992). Effects of the amphiphilic peptides melittin and mastoparan on calcium influx, phosphoinositide breakdown and arachidonic acid release in rat pheochromocytoma PC12 cells. J. Pharmacol. Exp. Ther. **260,** 369–375.

Coté, G.G., and Crain, R.C. (1993). Biochemistry of phosphoinositides. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44,** 333–356.

Drøbak, B.K. (1992). The plant phosphoinositide system. Biochem. J. **288,** 697–712.

Drøbak, B.K., and Ferguson, I.B. (1985). Release of Ca^{2+} from plant hypocotyl microsomes by inositol 1,4,5-trisphosphate. Biochem. Biophys. Res. Commun. **130,** 1241–1246.

Drøbak, B.K., and Watkins, P.A.C. (1994). Inositol (1,4,5)-trisphosphate production in plant cells: Stimulation by the venom peptides, melittin and mastoparan. Biochem. Biophys. Res. Commun. **205,** 739–745.

Drøbak, B.K., Watkins, P.A.C., Chattaway, J.A., Roberts, K., and Dawson, A.P. (1991). Metabolism of inositol (1,4,5)-trisphosphate by a soluble enzyme fraction from pea (*Pisum sativum*) roots. Plant Physiol. **95,** 412–419.

Drøbak, B.K., Watkins, P.A.C., Valenta, R., Dove, S.K., Lloyd, C.W., and Staiger, C.J. (1994). Inhibition of a plant plasma membrane phosphoinositide phospholipase C by the actin-binding protein, profilin. Plant J. **6,** 389–400.

Drøbak, B.K., Dove, S.K., and Staiger, C.J. (1996). Inositides and plant signalling. In Plant Membrane Biology, I.M. Moller and P. Brodelius, eds (Oxford, UK: Clarendon Press), pp. 29–50.

Felle, H. (1988). Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. Planta **174,** 495–499.

Frank, T.M., and Fein, A. (1991). The role of the inositol phosphate cascade in visual excitation of invertebrate microvillar photoreceptors. J. Gen. Physiol. **97,** 697–723.

Franklin-Tong, V.E., Lawrence, M.J., and Franklin, F.C.H. (1988). An in vitro bioassay for the stigmatic product of the self-incompatibility gene in *P. rhoeas* L. New Phytol. **110,** 109–118.

Franklin-Tong, V.E., Ride, J.P., Read, N.D., Trewavas, A.J., and Franklin, F.C.H. (1993). The self-incompatibility response in *P. rhoeas* is mediated by cytosolic free calcium. Plant J. **4,** 163–177.

Franklin-Tong, V.E., Ride, J.P., and Franklin, F.C.H. (1995). Recombinant stigmatic self-incompatibility (S-) protein elicits a Ca^{2+} transient in pollen of *P. rhoeas*. Plant J. **8,** 299–307.

Ghosh, T.K., Eis, P.S., Mullaney, J.M., Ebert, C.L., and Gill, D.L. (1988). Competitive, reversible and potent antagonism of inositol 1,4,5-trisphosphate–activated calcium release by heparin. J. Biol. Chem. **263,** 11075–11079.

Gilkey, J.C., Jaffe, L.F., Ridgway, E.B., and Reynolds, G.T. (1978). A free calcium wave traverses the activating egg of the medaka, *Oryzias latipes*. J. Cell Biol. **76,** 448–466.

Gilroy, S., Read, N.D., and Trewavas, A.J. (1990). Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. Nature **346,** 769–771.

Gilroy, S., Bethke, P.C., and Jones, R.L. (1993). Calcium homeostasis in plants. J. Cell Sci. **106,** 453–462.

Helsper, J.P.F.G., de Groot, P.F.M., Linskens, H.F., and Jackson, J.F. (1986). Phosphatidylinositol phospholipase C activity in pollen of *Lilium longiflorum*. Phytochemistry **25,** 2053–2055.

Helsper, J.P.F.G., Heemskerk, J.W.M., and Veerkamp, J.H. (1987). Cytosolic and particulate phosphotidylinositol phospholipase C activities in pollen tubes of *Lilium longiflorum*. Plant Physiol. **71,** 120–126.

Hepler, P.K., Palevitz, B.A., Lancelle, S.A., McCauley, M.M., and Lichtscheidel, I. (1990). Cortical endoplasmic reticulum in plants. J. Cell Sci. **96,** 355–373.

Heslop-Harrison, J., and Heslop-Harrison, Y. (1990). Dynamic aspects of apical zonation in the angiosperm pollen tube. Sex. Plant Repro. **3,** 187–194.

Hetherington, A.M., and Drøbak, B.K. (1992). Inositol-containing lipids in higher plants. Prog. Lipid Res. **31,** 53–63.

Hirayama, T., Ohto, C., Mizoguchi, T., and Shinozaki, K. (1995). A gene encoding a phosphatidylinositol-specific phospholipase C induced by dehydration and salt stress in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **92**, 3903–3907.

Huang, C.-H., Tate, B.F., Crain, R.C., and Coté, G.G. (1995). Multiple phosphoinositide-specific phospholipases C in oat roots: Characterization and partial purification. Plant J. **8**, 257–267.

Iino, M., and Endo, M. (1992). Calcium-dependent feedback control of inositol 1,4,5-trisphosphate–induced Ca^{2+} release. Nature **360**, 76–78.

Iwanami, Y. (1956). Protoplasmic movement in pollen grains and tubes. Phytomorphology **6**, 288–296.

Jaffe, L.A. (1993). Classes and mechanisms of calcium waves. Cell Calcium **14**, 736–745.

Jaffe, L.A., Weisenseel, M.H., and Jaffe, L.F. (1975). Calcium accumulation within the growing tips of pollen tubes. J. Cell Biol. **67**, 488–492.

Joseph, S.K., Esch, T., and Bonner, W.D. (1989). Hydrolysis of inositol by plant cell extracts. Biochem. J. **264**, 851–856.

Jouaville, L.S., Ichas, F., Holmuhamedov, E.L., Camacho, P., and Lechleiter, J.D. (1995). Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes. Nature **377**, 438–441.

Kobayashi, S., Somlyo, A., and Somlyo, A.P. (1988). Heparin inhibits the inositol 1,4,5-trisphosphate–dependent, but not the independent, calcium release induced by guanine nucleotide in vascular smooth tissue. Biochem. Biophys. Res. Commun. **153**, 625–631.

Krauss-Friedmann, N. (1994). Signal transduction and calcium: A suggested role for the cytoskeleton in the inositol 1,4,5-trisphosphate action. Cell Motil. Cytoskeleton **28**, 279–284.

Kuno, M., and Gardner, P. (1987). Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. Nature **326**, 301–304.

Legendre, L., Yuen, Y.G., Crain, R., Haddock, N., Heinstein, P.F., and Low, P.S. (1993). Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. J. Biol. Chem. **268**, 24559–24563.

Malhó, R., Read, N.D., Pais, M.S., and Trewavas, A.J. (1994). Role of cytosolic free calcium in the reorientation of pollen tube growth. Plant J. **5**, 331–341.

Malhó, R., Read, N.D., Trewavas, A.J., and Pais, M.S. (1995). Calcium channel activity during pollen tube growth and reorientation. Plant Cell **7**, 1173–1184.

McAinsh, M.R., Brownlee, C., and Hetherington, A.M. (1992). Visualizing changes in cytosolic free Ca^{2+} during the response of stomatal guard cells to abscisic acid. Plant Cell **4**, 1113–1122.

McAinsh, M.R., Webb, A.A.R., Taylor, J.E., and Hetherington, A.M. (1995). Stimulus-induced oscillations in guard cell cytosolic free Ca^{2+}. Plant Cell **7**, 1207–1219.

Melin, P.M., Pical, C., Jergil, B., and Sommarin, M. (1992). Polyphosphoinositide phospholipase C in wheat root plasma-membranes—Partial purification and characterization. Biochim. Biophys. Acta **1123**, 163–169.

Michelangeli, F., Mezna, M., Tovey, S., and Sayers, L.G. (1995). Pharmacological modulators of the inositol 1,4,5-trisphosphate receptor. Neuropharmacology **34**, 1111–1122.

Miller, D.D., Callaham, D.A., Gross, D.J., and Hepler, P.K. (1992). Free Ca^{2+} gradient in growing pollen tubes of *Lilium*. J. Cell Sci. **101**, 7–12.

Miyazaki, S., Yukazaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S., and Mikoshiba, K. (1992). Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. Science **257**, 251–255.

Mohri, T., Ivonnet, P.I., and Chambers, E.L. (1995). Effect on sperm-induced activation current and increase of cytosolic Ca^{2+} by agents that modify the mobilization of $[Ca^{2+}]_i$. I. Heparin and pentosan polysulfate. Dev. Biol. **172**, 139–157.

Obermeyer, G., and Weisenseel, M.H. (1991). Calcium channel blocker and calmodulin antagonists affect the gradient of free calcium ions in lily pollen tubes. Eur. J. Cell Biol. **56**, 319–327.

O'Driscoll, D., Read, S.M., and Steer, M.W. (1993). Determination of cell wall porosity by microscopy: Walls of cultured cells and pollen tubes. Acta Bot. Neerl. **42**, 237–244.

Palmer, S., and Wakelam, M.J.O. (1990). Mass measurement of inositol 1,4,5-trisphosphate using a specific binding assay. In Methods in Inositide Reseach, R.F. Irvine, ed (New York: Raven Press), pp. 127–134.

Picton, J.M., and Steer, M.W. (1983). Evidence for the role of calcium ions in tip extension in pollen tubes. Protoplasma **115**, 11–17.

Pierson, E.S., Miller, D.D., Callaham, D.A., Shipley, A.M., Rivers, B.A., Cresti, M., and Hepler, P.K. (1994). Pollen tube growth is coupled to the extracellular calcium ion flux and the intracellular calcium gradient: Effect of BAPTA-type buffers and hypotonic media. Plant Cell **6**, 1815–1828.

Prentki, M., Deeney, J.T., Matschinsky, F.M., and Joseph, S.K. (1986). Neomycin: A specific drug to study inositol lipid signalling system? FEBS Lett. **197**, 285–288.

Quarmby, L.M., Yueh, Y.G., Cheshire, J.L., Snell, W.J., and Crain, R.C. (1992). Inositol phospholipid metabolism may trigger flagellar excision in *Chlamydomonas reinhardtii*. J. Cell Biol. **116**, 737–744.

Ranjeva, R., Carrasco, A., and Boudet, A.M. (1988). Inositol trisphosphate stimulates the release of calcium from intact vacuoles isolated from *Acer* cells. FEBS Lett. **230**, 137–141.

Rathore, K.S., Cork, R.J., and Robinson, K.R. (1991). A cytoplasmic gradient of Ca^{2+} is correlated with the growth of lily pollen tubes. Dev. Biol. **148**, 612–619.

Reiss, H.-D., and Herth, W. (1985). Nifedipine-sensitive calcium channels are involved in polar growth of lily pollen tubes. J. Cell Sci. **76**, 247–254.

Rudd, J.J., Franklin, F.C.H., Lord, J.M., and Franklin-Tong, V.E. (1996). Increased phosphorylation of a 26-kD pollen protein is induced by the self-incompatibility response in *Papaver rhoeas*. Plant Cell **8**, 713–724.

Sayers, L.G., and Michelangeli, F. (1993). The inhibition of the inositol 1,4,5-trisphosphate receptor from rat cerebellum by spermine and other polyamines. Biochem. Biophys. Res. Commun. **197**, 1203–1208.

Schumaker, K., and Sze, H. (1987). Inositol 1,4,5-trisphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat roots. J. Biol. Chem. **262**, 3944–3946.

Shacklock, P.S., Read, N.D., and Trewavas, A.J. (1992). Cytosolic free calcium mediates red light–induced photomorphogenesis. Nature **358**, 753–755.

Steer, M.W., and Steer, J.M. (1989). Pollen tube tip growth. New Phytol. **111**, 323–358.

Trewavas, A.J., and Gilroy, S. (1991). Signal transduction in plant cells. Trends Genet. **7**, 356–361.

Wallace, M.A., and Carter, H.R. (1989). Effects of wasp venom peptide, mastoparan, on a phosphoinositide-specific phospholipase C purified from rabbit brain membranes. Biochim. Biophys. Acta **1006,** 311–316.

Wang, J.P., Needleman, D.H., Seryshev, A.B., Aghdasi, B., Slavik, K.J., Liu, S.Q., Pedersen, S.E., and Hamilton, S.L. (1996). Interaction between ryanodine and neomycin binding sites on Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. **271,** 8387–8393.

Worley, P.F., Baraban, J.M., Supattapone, S., Wilson, V.S., and Snyder, S.H. (1987). Characterization of inositol 1,4,5-trisphosphate receptor binding in brain. J. Biol. Chem. **262,** 12132–12136.