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Calcium signalling in pollen of *Papaver rhoeas* undergoing the self-incompatibility (SI) response

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Abstract Self-incompatibility (SI) is a genetically controlled system used by many flowering plants to prevent self-pollination. We established, using calcium imaging, that the SI response in *Papaver rhoeas* L. (poppy) pollen involves a Ca^{2+}-mediated intracellular signalling pathway. Here we review what is known about the signalling components and cascades implicated in the SI response in poppy pollen. We present some studies using calcium green (CG-1) that show SI-induced alterations in CG-1 fluorescence and localization. We have begun to examine potential sources of Ca^{2+} involved in the responses induced by SI. This work presents preliminary data showing that influx of extracellular Ca^{2+} at the "shank" of the pollen tube is possible. This is the first evidence suggesting that influx at this localization may play a role in the SI response. We also describe preliminary studies that begin to investigate whether the phosphoinositide signalling pathway is implicated in the SI response.

Keywords Calcium influx · Calcium signalling · Inositide signalling · Pollen · Self-incompatibility

Introduction

Self-incompatibility (SI) is a mechanism used by many flowering plants to prevent self-pollination. In *Papaver rhoeas* L., var. Shirley, SI is controlled by a single, multiallelic, gametophytically controlled *S* gene (Lawrence et al. 1978). Several alleles of the stigmatic *S* gene have been cloned (Foote et al. 1994; Walker et al. 1996; Kurup et al. 1998). We recently established that certain residues are crucial for the recognition of S_1 pollen (Kakeda et al. 1998; Jordan et al. 1999), although the exact molecular basis of allelic specificity remains to be elucidated. Identification of the pollen *S*-receptor is an important challenge currently being pursued. We have been investigating the intracellular signal transduction components and cascades involved in eliciting the SI response in *P. rhoeas* pollen. What is emerging is a network of components, signals and responses triggered as a consequence of the stigmatic S proteins interacting with incompatible pollen during the SI response (for recent reviews see Wheeler et al. 1999; Jordan et al. 2000a).

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Ca^{2+} as an SI signal transducer, and its targets

The first intracellular signalling component identified in the *P. rhoeas* SI signal transduction cascade was cytosolic free calcium (Ca^{2+}_i). The SI response activates increases in $[Ca^{2+}]_i$ within a few seconds of an incompatible interaction (Franklin-Tong et al. 1993, 1995, 1997). Confocal imaging of pollen tubes microinjected with calcium green-1 (CG-1) provided the first evidence that $[Ca^{2+}]_i$ plays a key role in mediating inhibition of *Papaver* pollen tube growth through the SI reaction (Franklin-Tong et al. 1993), with incompatible S proteins acting as signal molecules (Franklin-Tong et al. 1995). Subsequently, ratiometric Ca^{2+}-imaging using Fura-2 dextran provided more detailed information and accurate calibration of $[Ca^{2+}]_i$ in *P. rhoeas* pollen tubes undergoing the SI response (Franklin-Tong et al. 1997). Together, these data implicated a Ca^{2+}-mediated intracellular signalling pathway in SI-induced pollen tube inhibition. Since it is well established that Ca^{2+}_i acts as a second messenger in plant cells and that Ca^{2+} is important for the regulation of pollen tube growth (for recent reviews see Franklin-Tong 1999a,b; Rudd and Franklin-Tong 1999), this is consistent with expectations.

We are interested in the targets of the Ca^{2+} signals, and have established that several protein kinases are involved in SI in *Papaver* pollen. A change in the phos-

phorylation state of proteins resulting from the activation of protein kinases is one of the most important molecular mechanisms by which cells respond to extracellular signals. We have evidence for the SI response triggering the S-specific phosphorylation of several pollen proteins. Two of these are p26.1 and p68 (Rudd et al. 1996, 1997). Phosphorylation of p26.1 is Ca^{2+}-dependent and involves a Ca^{2+}-dependent protein kinase. The recent cloning of p26.1 indicates that it is an inorganic pyrophosphatase (J.J. Rudd, V.E. Franklin-Tong, F.C.H. Franklin, unpublished data). We also recently identified p52, which is a mitogen-activated protein kinase (MAPK) activity stimulated during the SI response in incompatible pollen, downstream of increases in $[Ca^{2+}]_i$ (J.J. Rudd, F.C.H. Franklin, and V.E. Franklin-Tong, unpublished data).

Recently, we established that the actin cytoskeleton is a target for the SI-induced signalling cascade(s). Within about 1–2 min of SI-induction, F-actin is detected at the pollen tube tip, F-actin bundles are detected adjacent to the plasma membrane, and actin bundles in the lumen appear to be fragmented. From 20 min to several hours later, F-actin was organised into punctate foci, which enlarged over this time period (Geitmann et al. 2000; Snowman et al. 2000a,b). Downstream of these events, we identified *S*-specific nuclear DNA fragmentation that was detected 4–12 h after induction of the SI response (Jordan et al. 2000b). Nuclear DNA fragmentation is a hallmark feature of programmed cell death (PCD) and is a key target for PCD signalling cascades. These findings suggest that PCD is triggered by the SI response in *Papaver* pollen, and is likely to represent the endpoint in the signalling cascade.

Here we describe work that begins to examine the potential sources of Ca^{2+} involved in the responses induced by SI, using a variety of approaches.

Materials and methods

Growth of pollen and induction of SI in vitro

Pollen from plants of *P. rhoeas* L. (var. Shirley) segregating for known incompatibility genotypes were used. Pollen was grown using an in vitro system, adapted from Franklin-Tong et al. (1993), using solidified germination medium (GM). For the vibrating probe work, the $[Ca^{2+}]$ in GM was lowered to 50 µM and growing pollen was covered with 300 µl liquid germination medium. Recombinant S proteins (see Kakeda et al. 1998, for full details) were used to challenge incompatible pollen and compatible pollen to induce an SI response.

CG-1 imaging

Pollen tubes that had grown to approximately 100 µm long were loaded for 15 min with 30 µM CG-acetoxymethyl ester, and then washed. Once growth had recommenced (a few minutes later) the tubes were challenged with S proteins and imaged immediately. Confocal laser scanning microscopy was performed using a Nikon Diaphot TMD inverted microscope attached to a Bio-Rad MRC-600 confocal system equipped with a 25 mW argon-ion laser and a Nikon PlanApo 60x oil immersion objective. CG fluorescence was detected using standard FITC filters.

Calcium-selective vibrating probe measurements

Measurements of Ca^{2+}-fluxes around pollen grains and tubes were made using Ca^{2+} selective vibrating probes (see Smith et al. 1994). Two-dimensional scans allowed mapping of both the magnitude and vector direction of the Ca^{2+} fluxes. Matlab scripts were used to place vectors on images with appropriate scale bars. Data matrices produced by the ion-selective probe software, as described in Holdaway-Clarke et al. (1997), were analyzed using an MS Excel spreadsheet to convert data from the background-mV estimation of concentration and microvolt-difference estimation of the local gradient into specific ion flux (pmole cm^{-2} sec^{-1}). A Nernst slope and intercept was determined for each electrode used. Calibration of the efficiency of the probe in a dynamic state was performed using point sources of Ca^{2+} as described by Smith et al. (1994). The background mV values a distance from and near the tube were measured prior to each series of measurements.

Inositide labelling and inositol analysis of pollen

Pollen was grown in the presence of 10 µCi ml^{-1} [2-^{3}H]-inositol for at least 3 h to obtain good incorporation of labeled inositol into the pollen. Extracts from growing pollen tubes were made as described for yeast cells (Dove et al. 1997). We produced deacylated derivatives of the phosphoinositides to identify the glycerophosphoinositol esters (Gro*P*InsP_ns). Gro*P*InsP_ns were separated by HPLC as described by Pical et al. (1999). Using known ^{14}C-labelled Gro*P*InsP_ns from yeast as standards, we were able to identify the phosphatidylinositols present in ^{3}H-inositol-labelled pollen. ^{3}H-inositol-labelled pollen was also used for SI challenges. Samples were taken at various time points, and the lipid fraction extracted and analysed for alterations in inositide level using HPLC. Each experiment was carried out with at least two independent samples.

Results

Spatial localization of increases in $[Ca^{2+}]_i$ triggered by the SI response

Fig. 1a–c shows three low magnification confocal images of a pollen tube labelled with CG-acetoxymethyl ester (CG-1), in which an incompatible SI response has been induced, to indicate the region of the pollen tube in which the localized alterations in fluorescence are detected. As with previous imaging with CG-1, a faint signal is detectable in the region where increases in $[Ca^{2+}]_i$ are observed before SI treatment (Fig. 1a). Increases in fluorescence detected in the first few minutes of the SI response are more spatially localized than previously thought, and high fluorescence appears to be associated with, or in the form of, thin tracks (Fig. 1b). Confocal imaging reveals that the high fluorescence in this region has a reticulate pattern (Fig. 1d, e; 2 and 5 min after challenge, respectively). Later increases in fluorescence are are more extensive, spreading though a much larger area of the cell (Fig. 1c), but distinct strand-like patterns of high fluorescence are still detected. Given the reticulate morphology of the fluorescence signal and the fact that acetoxymethyl ester dyes have a tendency to compartmentalize, we think that much of the signal results from sequestration of the dye into organelles or inclusions, or to a combination of enhanced dye sequestration

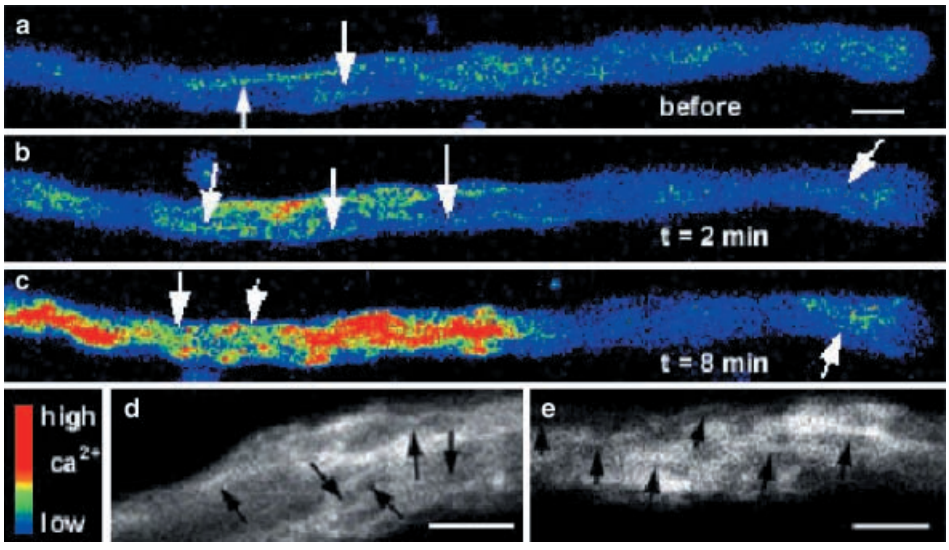

Fig. 1a–e CG-1 imaging in pollen tubes of *Papaver rhoeas* using calcium green-1. **a** Untreated pollen tube imaged 1 min before SI-treatment using CG-1. Part of the CG-1 signal is likely to be partially sequestered dye (see the faint fine strands indicated by *arrows*). **b** The pollen tube in **a** 2 min after incompatible SI treatment. The CG-1 signal reveals a transient increase in fluorescence. These are highly localized and are visualized as long strands (*arrows*). **c** The same pollen tube as in **a** and **b** 8 min after SI treatment. Increases in fluorescence are more extensive; distinct strand-like patterns are still detected (*arrows*). **d, e** Incompatible pollen tubes imaged 2 and 5 min after SI treatment, respectively. Regions of high CG-1 fluorescence have a reticulate form. *Arrows* indicate the thin, thread-like areas where fluorescence is very locally increased. **a**, **b**, and **c** are shown in *pseudocolour* scale for relative alterations in CG-1 fluorescence. **d** and **e** are shown in a *grey* scale, with increasing *white* level indicating higher fluorescence. *Bars* 70 µm

as well as Ca^{2+} uptake. However, we cannot rule out that some of the CG-1 signal may derive from alterations in $[Ca^{2+}]_i$. These observations raise the possibility that the SI treatment increases the uptake of anions, e.g., CG-1, and Ca^{2+} into sub-cellular compartments. It would be highly speculative to suggest what these inclusions might be until information on the organelles associated with this region of the pollen tube is available. However, from the visual evidence, we might guess that vacuoles or the endoplasmic reticulum might potentially be involved.

Measurements of Ca^{2+} influx in *P. rhoeas* pollen

Although we have established that there are increases in $[Ca^{2+}]_i$ stimulated by the SI response, we had not previously investigated the possible source(s) of Ca^{2+} involved. To examine the possibility of extracellular Ca^{2+} (Ca^{2+}_e) involvement, we recently used the calcium-selective vibrating probe to map influxes of calcium in germinating pollen grains and growing pollen tubes of *P. rhoeas*. In ungerminated and germinating pollen grains we detected clear evidence for Ca^{2+} influx (n=16) (data not shown). In order to obtain a better idea of the overall patterns of the Ca^{2+} fluxes, two-dimensional scans were made of both ungerminated pollen grains (n=3) and pollen grains that had just germinated (n=5). The vector plots in Fig. 2 show typical examples of the Ca^{2+} fluxes detected using this method. Fig. 2a shows the Ca^{2+} fluxes during germination. There is a large Ca^{2+} influx of about 10 pmole cm^{-2} sec^{-1} at the bottom right hand side of the pollen grain, where one of the colpal apertures is located. There is also a Ca^{2+} influx of about 10 pmole cm^{-2} sec^{-1} directed at the base of the emerging pollen tube. Interestingly, the largest fluxes are not directed at the pollen tube tip, as expected. This is likely to be a phenomenon that is found in newly germinating pollen tubes, since these pollen tubes were all very short. Longer pollen tubes of this species, and several other studies on long pollen tubes of other species have demonstrated that the largest influx is at the extreme tip (Malhó et al. 1995; Pierson et al.1996).

We have used the Ca^{2+}-selective vibrating probe to ascertain that, as with all other species examined, there is influx at the tip of growing pollen tubes of *P. rhoeas* (data not shown; V.E. Franklin-Tong, T. Holdaway-Clarke, K.R. Straatman, J.G. Kunkel, P.K.Hepler, unpublished data). We have also measured Ca^{2+} fluxes along the shanks of growing pollen tubes of *P. rhoeas*, since this was the region of the pollen tube where the increases in $[Ca^{2+}]_i$ were detected in the SI response. Fig. 2b shows a two-dimensional map of Ca^{2+} influxes in a growing pollen tube shank, which shows large extracellular Ca^{2+} fluxes in the "shank" region. In Fig. 2b, a large inwardly-directed Ca^{2+} flux of 5.0 pmol cm^{-2} sec^{-1} and a large outwardly-directed flux of 7.8 pmol cm^{-2} sec^{-1} was measured in the region where the male germ unit was located. We also performed one-dimensional measurements of Ca^{2+} fluxes along the shanks of growing pollen tubes of *P. rhoeas* that were several hundred µm long (n=18) (Fig. 2c). The Ca^{2+} fluxes detected along the pollen tube shanks were highly dynamic and varied substantially. Nevertheless, we consistently detected Ca^{2+} influxes of

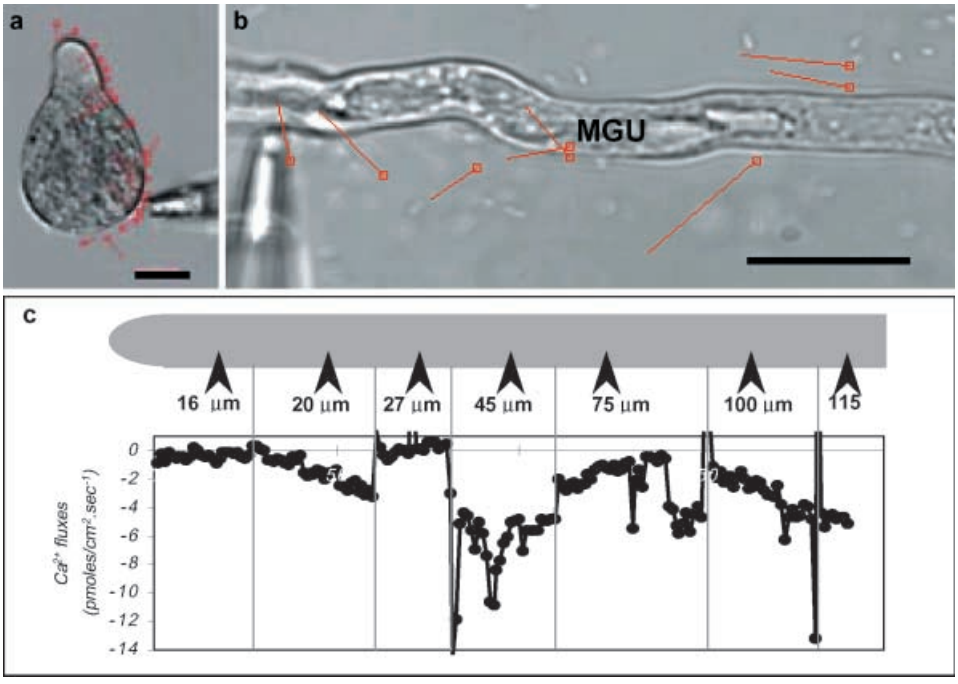

Fig. 2a–c Calcium fluxes around pollen grains and tubes of *Papaver rhoeas*. Using the vibrating probe, we measured Ca^{2+} fixes adjacent to pollen grains and pollen tubes. Vector plots of Ca^{2+} fluxes in (**a**) and (**b**) are indicated by *red lines* emanating from the site of measurement (*box*). The *lines* indicate both the direction and the magnitude of the Ca^{2+} fluxes. The *calibration bar* in each *block* is 10 pmol cm^{-2} sec^{-1}. **a** A pollen tube emerging from a pollen grain of *P. rhoeas*. Ca^{2+} influx is detected at the colpal aperture (adjacent to the probe tip). Note that the largest influxes detected are at the base of the emerging pollen tube, rather than at the tip. **b** Ca^{2+} fluxes in a typical growing pollen tube shank of *P. rhoeas*. Ca^{2+} fluxes can be detected around the shanks of a growing pollen tube. *MGU* indicates the male germ unit. *Bar* 10µm. **c** Representative plot of Ca^{2+} fluxes along an individual growing pollen tube shank of *P. rhoeas*. Measurements of Ca^{2+} flux were made at various points along the pollen tube shank (indicated on the representation of the *pollen tube* by the *arrow heads* and the *distance* behind the tip in µm). These were recorded sequentially at a particular point along the pollen tube, and the probe then moved to another location. The plot shows net Ca^{2+} flux in pmol cm^{-2} sec^{-1} (Jo), with net influx as a negative reading. There is clearly evidence for net Ca^{2+} influx at some points along the pollen tube shank.

between 1 to 9 pmol cm^{-2} sec^{-1} in the region about 50 µm behind the pollen tube tip. Since this was of the same order as influxes measured at the pollen tube tips, they are not insignificant, and provide good evidence for the presence of Ca^{2+} influx in regions of the pollen tube other than the pollen tube tip. This represents (to our knowledge) the first demonstration of large extracellular Ca^{2+} currents in the "shank" region (V.E. Franklin-Tong, T. Holdaway-Clarke, K.R. Straatman, J.G. Kunkel, P.K.Hepler, unpublished data), since most of the pollen literature appears to consider that there is no or negligible Ca^{2+} influx at the pollen tube except for at the immediate tip.

Our finding that Ca^{2+} influx does occur around pollen grains is important, because in vivo, incompatible pollen undergoing the SI response in *Papaver* is inhibited prior to, or soon after, germination, but after polarity has been determined (i.e. before any appreciable length of pollen tube is obtained). Since Ca^{2+} fluxes are present around the pollen grains before and during germination, this indicates that an incompatible SI challenge has something to "work with" with regard to altering the normal patterns of Ca^{2+} flux in the cell. The reason why we performed $[Ca^{2+}]_i$ imaging on pollen tubes is because pollen grains are difficult to microinject. Moreover, since the SI response may be elicited at any time up to several hours after germination, imaging pollen tubes also had the advantage that the organelles were spatially separated and more easily distinguishable. Since Ca^{2+} imaging unexpectedly revealed alterations in $[Ca^{2+}]_i$ in the shank region of the pollen tube during the SI response, we wondered if this might be attributable to Ca^{2+} influx. These preliminary studies provide good evidence for Ca^{2+} influx at the shank of the pollen tube, which clearly indicates the potential for Ca^{2+} influx in this region. Furthermore, we have recently used these techniques to demonstrate that the SI response involves an influx of extracellular Ca^{2+} and, furthermore, that the increases occur along the shank of the tube (V.E. Franklin-Tong, T. Holdaway-Clarke, K.R. Straatman, J.G. Kunkel, and P.K. Hepler, unpublished data).

Is inositol phosphate signalling involved in SI?

Although extracellular Ca^{2+} is implicated in regulating pollen tube growth, our detection of Ca^{2+} waves with imaging suggests that intracellular Ca^{2+} stores play an important role in the SI response. Since Ca^{2+} cannot itself move any distance, two possibilities whereby this might

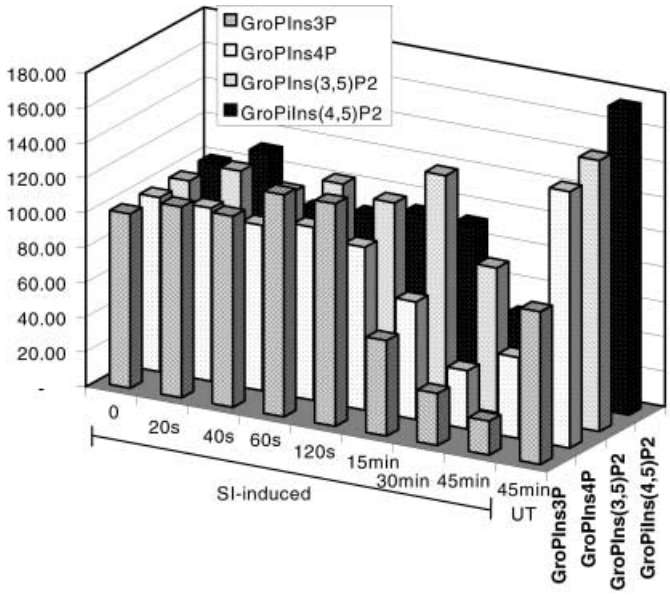

Fig. 3 Effect of SI-challenge on some inositol lipids in growing pollen tubes. ^{3}H-inositol-labelled pollen tubes were given a SI treatment by addition of recombinant incompatible S-proteins (S_1e and S_3e). SI-induced pollen tubes were collected at the time intervals indicated and deacylated phospholipids were analysed using HPLC. Peaks were identified using known ^{14}C labelled inositol lipids derived from yeast. The controls shown here comprised untreated pollen tubes; compatible controls were also made (data not shown). The histogram shows the percentage change in inositol lipid components relative to t=0 (which is taken as 100%) for each time point. This shows the mean data from two independent experiments

be achieved are Ca^{2+}-induced Ca^{2+} release (CICR) or inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3)-induced Ca^{2+} release (IICR). We previously showed that regulation of *P. rhoeas* pollen tube growth involves Ins(1,4,5)P_3-induced Ca^{2+} release and Ca^{2+} waves (Franklin-Tong et al. 1996), thereby implicating the involvement of a functional phosphoinositide system in pollen tube inhibition. Since, to date, a role for the phosphoinositide-signalling pathway in the SI response has not been investigated, we have initiated studies to investigate this possibility.

We first attempted to establish which of the phosphatidylinositols could be identified in pollen labelled with [2-^{3}H] inositol. Using ^{14}C-labelled probes as standards, we provisionally identified several phosphatidylinositols by their glycerophosphoinositol esters, which are deacylated derivatives of the phosphoinositides (e.g. Gro*P*Ins3*P* is the deacylated form of PtdIns3*P*). They are: Gro*P*Ins3*P*, Gro*P*Ins4*P*, Gro*P*Ins(3,5)P_2 and Gro*P*Ins(4,5)P_2 (data not shown). A peak which may correspond to Gro*P*Ins(3,4)P_2 was also identified in some samples. We found that although the ratio Gro*P*Ins(3,5)P_2: Gro*P*Ins(4,5)P_2 varied between experiments (1:4 to 1:2), it was always relatively high. Although this identification is not totally conclusive, it is unlikely to be wrong and represents the first report of this type in pollen.

Having established that we could identify at least some of the phosphoinositides in pollen, we performed SI reactions in vitro using ^{3}H-inositol-labelled pollen, taking samples at various time points. A summary of the alterations in the lipid fraction analysed using HPLC is shown in Fig. 3. The time points at which samples were taken were chosen in two ranges. One was a time series taken at 0, 20, 40, 60, and 120 s, which would hopefully pick up any rapid alterations in inositol lipid turnover. The other time series was taken at longer time intervals (15, 30, and 45 min) that might indicate any slower or later changes in inositide turnover.

Fig. 3 shows that although there are changes in the levels of some lipids, in particular for Gro*P*Ins(4,5)P_2 and Gro*P*Ins(3,5)P_2, there are no striking alterations in these samples. However, the data do show an overall 20% decrease in labelled Gro*P*Ins(4,5)P_2 40 s after incompatible SI challenge. Since PtdIns(4,5)P_2 is a target for phosphoinositidase C activity, generating Ins(1,4,5)P_3 and diacyl glycerol, this decrease in Gro*P*Ins(4,5)P_2 could potentially indicate the production of Ins(1,4,5)P_3. One striking difference detected in incompatible pollen tubes was that 15–30 min after SI induction the levels of all four inositol lipids examined were much reduced. Comparisons with unchallenged pollen shows that SI-challenged pollen tubes had only about 25–30% of the levels of incorporation of ^{3}H-inositol found in unstimulated cells. The significance of this is not known, as the incorporation of ^{3}H-inositol continues even after tube growth stops. As the pollen tubes are unlikely to be labelled to equilibrium, it is difficult to say is what is happening to them in terms of changes in mass. However, this does give the first indication that there may be changes in inositide levels in the SI response. Further studies on this pathway, and other potential Ca^{2+}–releasing mechanisms, will be important for ascertaining the Ca^{2+} stores utilized in the SI response.

In conclusion, we have data that indicate alterations in [Ca^{2+}] in incompatible pollen tubes undergoing the SI response. Here we introduce the idea that dye/Ca^{2+} uptake into cytoplasmic compartments is also stimulated by SI-treatment. On the question of pools of Ca^{2+} involved in the SI response, from current data it seems quite feasible that both extracellular and intracellular Ca^{2+} are involved in SI signalling. Why the “shank” region of the tube is involved is still not known. This awaits information on the organization of organelles in these pollen tubes. It might be postulated that there are S-receptors localised in this region of the pollen tube, possibly linked to Ca^{2+} channels, which allows Ca^{2+} influx in this region. This might stimulate intracellular Ca^{2+} release, which could then transduce signals to the pollen tube tip. This provides a testable hypothesis that can be explored in future studies.

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