Role of phosphatidic acid during differentiation of *Phytophthora palmivora* zoospores

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Zoospores of the fungus *Phytophthora palmivora* undergoing synchronous differentiation showed a rapid increase (250%) in phosphatidic acid (PA) concentration within 20 s of the inducing stimulus. There were only small (<100%) changes in cGMP and cAMP and inositol phosphates during the same period. There was no consistent change in the concentration of diacyl glycerols during the first five minutes of differentiation. The addition of exogenous PA (3 µM) induced zoospore differentiation, with the optimum concentration dependent upon the Ca²⁺ concentration in the suspension medium. Other phospholipids were ineffective as inducers. Both PA production and spore differentiation were Ca²⁺-dependent, and the addition of the Ca²⁺ channel blocker verapamil, or the removal of exogenous Ca²⁺ by EGTA addition, both reduced PA accumulation and slowed differentiation. We suggest that PA production in this organism arises via a stimulus-activated phospholipase D, and may act as a second messenger. There is no evidence for any role for cyclic nucleotides or inositol phosphates as second messengers during the early events of zoospore differentiation in this species.

Introduction

Fungi of the family Pythiaceae infect their hosts through motile, wall-less cells termed zoospores (Carlile, 1986). These are attracted to host surfaces by chemotaxis (Cameron & Carlile, 1978; Goldberg *et al.*, 1989). In order to infect the host, the motile spore must change to a germling, with a transient intermediate, the cyst or cystospore. This transition from zoospore to germling takes less than 30 min at 25 °C, while encystment is completed in less than 5 min. Encystment and germination are induced by contact with polysaccharides at the host surface (Hinch & Clarke, 1980; Irving & Grant, 1984; Longman & Callow, 1987; Estrada-Garcia *et al.*, 1990). They may also be induced by localized concentrations of Ca²⁺, which have been shown to occur on root surfaces (Miller & Gow, 1989).

Axenic suspensions of zoospores of *Phytophthora*

palmivora and *Phytophthora cinnamomi* undergo this transition in synchrony, in response to a variety of external stimuli, including pectate (Grant *et al.*, 1985), millimolar concentrations of Ca²⁺ or Sr²⁺ (Byrt *et al.*, 1982), or vigorous agitation (Tokunaga & Bartnicki-Garcia, 1971). Differentiation is dependent upon low concentrations of Ca²⁺ (Griffith *et al.*, 1988) and is accompanied by increases in the transmembrane fluxes of both this ion (Irving *et al.*, 1984) and Na⁺ (Iser *et al.*, 1989). Zoospore differentiation is also sensitive to external pH (Griffith *et al.*, 1988).

The zoospore is committed to encystment, the first stage of the process, within 20–30 s of the stimulus, though many of the morphological and metabolic changes are not observed for several minutes (Paktitis *et al.*, 1986). Although progress has been made in delineating external signals which induce these changes, there is still little information on the means by which the signals are transmitted to the cell interior.

In this paper, we report changes in phosphatidic acid (PA) concentrations during the critical period when zoospores of *P. palmivora* become committed to encystment. There were small increases in cyclic nucleotide levels during this period, but no evidence that either cyclic nucleotides or the inositol phosphate pathway is involved in intracellular signalling during this critical early stage.

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Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DAG, diacylglycerol; GC/MS, gas chromatography–mass spectrometry; IP, *myo*-inositol-4-phosphate; IP₃, *myo*-inositol-1,4,5-trisphosphate; IP₄, *myo*-inositol-1,3,4,5-tetrakisphosphate; PA, phosphatidic acid; PLC, phospholipase C; PLD, phospholipase D; RIA, radioimmune assay; TMS, trimethylsilyl.

Methods

Growth conditions. *Phytophthora palmivora* ATCC 26286 was used throughout the study. The methods for maintenance of the culture and the production and manipulation of zoospores have been described previously (Griffith *et al.*, 1988). Under the conditions used in the experiment, the free Ca^{2+} in the zoospore suspension was calculated to be approx. $0.2 \mu\text{M}$.

Where necessary, spores were labelled with [^{32}P]phosphate (AAEC, Lucas Heights, NSW) or *myo*-[^3H]inositol (Amersham Aust.) by growing mycelium on V-8 juice agar to which either $270 \mu\text{Ci}$ [^{32}P]phosphate or $10 \mu\text{Ci}$ *myo*-[^3H]inositol were added per 20 ml of medium, prior to solidification of the agar. Phosphate and inositol concentrations in the medium were shown to be 0.9 mM and $80 \mu\text{M}$, respectively. Phosphate was determined by ion chromatography (Smillie *et al.*, 1988) and *myo*-inositol as the TMS ether by GC.

Induction of differentiation. All experiments were carried out at $25 \pm 2^\circ\text{C}$. Zoospores were normally induced to encyst and germinate by the addition of Tris-pectate (Grant *et al.*, 1985). In some experiments, addition of 3 mM-Sr^{2+} , or agitation of the spore suspension for 30 s on a Vortex mixer was used. Suspensions of zoospores contained 2×10^6 cells ml^{-1} . An equal volume of distilled water was added to controls. Where treatments required addition of compounds in ethanol solution, an ethanol control was also included, since ethanol itself induces slow differentiation (Iser *et al.*, 1989). Cells were removed at intervals, and killed by addition of ice-cold perchloric acid or chloroform/methanol as appropriate. Additional samples were taken to observe the stage of differentiation of the population.

Where differentiation was induced by dipalmitoyl PA addition, a chloroform solution of the lipid was taken to dryness in a glass centrifuge tube using a stream of nitrogen. Zoospore suspension medium was added, and the material was brought into suspension by sonication for twenty 1 min cycles, using an MSE ultrasonicator fitted with an $1/8''$ probe. The emulsion was cooled in ice for 1 min between each sonication cycle. The same procedure was used where diacylglycerol (DAG) suspensions were prepared.

Cyclic nucleotide analysis. Cyclic nucleotides were determined in 0.5 ml samples of spore suspension (10^6 cells) to which 0.125 ml ice-cold 35% (v/v) perchloric acid was added. Extracts were neutralized with K_2CO_3 , and the perchlorate removed by centrifugation. Supernatants were freeze-dried and the residue taken up in $600 \mu\text{l}$ 0.05 M -acetate buffer, pH 5.8, containing 0.01% thiomersal. cAMP was assayed by the competitive binding assay (Gilman, 1972) and cGMP by RIA assay (Steiner *et al.*, 1972). The first set of measurements used the [^3H]-labelled nucleotide, and was then confirmed using the more sensitive [^{125}I]-labelled nucleotide. Assay materials were provided as kits (Amersham Aust.). Controls using phosphodiesterase-treated samples were run simultaneously to estimate non-specific binding. The non-specific binding values have been subtracted from the data reported.

Inositol phosphate analysis. When inositol phosphates were measured by the RIA procedure (Palmer *et al.*, 1989), the extraction procedure was identical to that used for cyclic nucleotides, and a commercial kit (Amersham Aust.) was used for the determinations.

When measurements were made using zoospores labelled with ^3H -labelled inositol, 15 ml aliquots (1.5×10^7 cells) were used and extracted by the method of Hawkins *et al.* (1986). The neutralized perchloric acid extract was freeze-dried, and fractionated by anion-exchange chromatography (Berridge *et al.*, 1983). The tritium content of the fractions was measured by liquid scintillation and the mass of each inositol phosphate calculated from the specific activity of the inositol in the culture medium. The separation was authenticated by extracting a large sample (10^8 spores) and fractionating the aqueous extract by HPLC as described by Irvine *et al.* (1985). Non-radioactive

standards (Sigma) were added to increase the mass of inositol phosphates present. The location of the individual inositides was determined by counting the radioactivity present in the eluant. ATP, measured by absorbance at 254 nm , was used as an internal standard in the separation. Individual radioactive inositol phosphates separated in this manner were then refractionated using the anion-exchange procedure described above.

Lipid analysis. Phosphatidyl inositol phosphates were extracted from ^{32}P -labelled zoospores using ice-cold chloroform/methanol (Bligh & Dyer, 1959), followed by acidified chloroform/methanol (Berridge *et al.*, 1983). Lipids were fractionated on silica gel TLC plates using the method of Jolles *et al.* (1981) and phosphatidyl inositides were identified by R_f values (Rouser *et al.*, 1970). These compounds accounted for 1–3% of the total lipid phosphorus, with the PIP_2 accounting for $<0.01\%$.

Phosphatidic acid in the total lipid extract was initially identified by its R_f value in two dimensional TLC on silica gel plates (Merck) using the solvent systems of Rouser *et al.* (1970) and by co-chromatography with a dipalmitoyl PA standard (Sigma). Radioactive phospholipids were located by autoradiography using X-Omat RP film (Kodak) exposed for 72 h. After location, radioactive areas were cut from the TLC plate and the radioactivity quantified by liquid scintillation counting. PA was only a minor component of the total lipid phosphorus, always $<1\%$ of the total, and generally not distinguishable on autoradiographs of unstimulated spore phospholipids.

Analysis of the PA fraction. A single sample containing 2.5×10^8 spores was prepared, stimulated to differentiate, and after 5 min the total lipids extracted as described above. A separate sample of radioactive zoospore phospholipid sample, containing some 40000 c.p.m. of ^{32}P , was added and the mixture was separated using preparative silica gel TLC in two dimensions, with the putative PA located by autoradiography. Material with the R_f of PA was extracted in chloroform/methanol (1:1), dried under nitrogen, transferred to a small vial and saponified and the products neutralized with excess HCl. The HCl was removed by freeze-drying and the saponified mixture derivatized with BSTFA and separated by GLC as described by Dunstan *et al.* (1990).

Analysis of lipids by gas chromatography. The diacyl glycerols (DAGs) from whole lipid extracts were analysed as their TMS ethers, using a 12 m , 0.33 mm i.d., aluminium-clad capillary column (S.G.E. HT-08) in a GC fitted with a flame ionization detector. The methods are described in Dunstan *et al.* (1990). Identification was made by comparing retention times with those of authentic DAGs (dipalmitin, diolein and dicaprin, each a mixture of 1,2 and 1,3 isomers, from Sigma) run as standards, and by demonstrating that the peaks identified as DAGs disappeared following saponification of the lipid sample. Fatty acids were identified by retention times relative to authentic standards (Sigma).

Gas chromatography-mass spectrometry. The products from the saponification of the PA were separated using a Hewlett Packard 5890 gas chromatograph and detected using a Hewlett Packard series 5970 Mass Selective Detector (MS) as described by Dunstan *et al.* (1990). Limitations on the temperature of the transfer oven prevented the use of identical columns and programmes in GC and GC/MS and thus precluded the direct identification of the DAG isomers by GC/MS.

Statistics and reproducibility. The mean values for cyclic nucleotide and inositol polyphosphate concentrations in stimulated cells were compared with those in controls using Student's *t* test. In experiments where only single or duplicate samples could be analysed, experiments were repeated at least twice and usually three times. The data presented in each figure or table is representative of the overall results and where there was variation between experiments, this is noted in the text.

Results

Changes in cyclic nucleotides

In unstimulated spores, cAMP concentrations were in the region of 1 pmol per 10^6 cells (Table 1), and remained at this level for at least 25 min. Following pectate addition, cAMP concentrations rose to 1.6–2 pmol per 10^6 cells within 12 s, and at 4 min, reached a maximum of 3.3 pmol per 10^6 cells, before declining to the level of the unstimulated population between 5 and 8 min. Over a series of 10 experiments, the level of cAMP in unstimulated spores varied by as much as 100%, and the magnitude and the timing of the increase shown at 4 min in Table 1 also varied. However, an increase in cAMP in stimulated spores was always observed during the first two minutes and although small, was significant at the $P < 0.05$ level.

Addition of the labdane diterpene forskolin, (1–50 μ M) had no effect on cAMP concentration in zoospores and did not induce differentiation in either of two trials. The lack of forskolin activity in *P. palmivora* spores suggests either that their adenylate cyclase is insensitive to the drug, in marked contrast to its stimulatory effect on most mammalian adenylate cyclases (Laurenza *et al.*, 1989), or that the drug failed to penetrate the spore. Forskolin could not therefore be used to elevate the cAMP concentrations in the absence of an external stimulus, to determine whether this alone would induce differentiation.

When spore differentiation was induced by the addition of exogenous phosphatidic acid, the concentration of cAMP did not increase over 10 min, and remained constant at 2 pmol per 10^6 cells. In particular, the increase in cAMP at 4 min in pectate-treated cells did not occur. However, the concentration of 2 pmol per 10^6 cells observed in unstimulated cells was within the range observed in pectate-stimulated cells in other experiments (Table 1). As these controls did not undergo rapid differentiation, elevation of cAMP to this level seems insufficient to trigger the synchronous encystment and germination observed in populations treated with pectate or PA.

The concentration of cGMP in unstimulated zoospores was an order of magnitude lower than that of cAMP (0.15–0.19 pmol per 10^6 cells, Table 1). Mean cGMP concentration increased only slightly in unstimulated cell populations over more than 30 min. Pectate addition resulted in an increase in cGMP concentration within 12 s of stimulation, but the increase did not continue. The mean values of the controls drifted upward, and the difference between stimulated and control populations was not significantly different after

Table 1. Cyclic nucleotide levels in *P. palmivora* zoospores during differentiation

Each data point is the mean of values from four separate experiments. Figures are pmol per 10^6 cells. The values in brackets are the standard deviations. Values with an asterisk were significantly different ($P < 0.05$) from the corresponding control value.

Time (min)	cAMP		cGMP	
	Control	Pectate treated	Control	Pectate treated
0.0	1.0 (0.3)	–	0.15 (0.01)	–
0.2	–	1.6 (0.3)*	–	0.20 (0.02)*
0.3	–	–	–	0.21 (0.01)*
0.5	1.1 (0.3)	1.5 (0.1)*	0.15 (0.02)	0.20 (0.01)*
0.7	–	–	–	0.20 (0.03)
0.8	–	–	–	0.20 (0.04)
1.0	1.1 (0.2)	1.5 (0.2)*	0.16 (0.01)	0.23 (0.03)*
1.5	–	–	–	0.20 (0.03)
2.0	1.1 (0.2)	1.6 (0.2)*	0.18 (0.03)	0.22 (0.02)*
4.0	1.0 (0.1)	3.3 (1.0)*	–	0.20 (0.03)
5.0	1.0 (0.4)	1.8 (1.3)	0.17 (0.04)	0.19 (0.01)
8.0	1.0 (0.2)	1.2 (0.2)	0.18 (0.03)	0.21 (0.05)
10.0	1.0 (0.3)	0.9 (0.5)	0.18 (0.04)	0.21 (0.03)
15.0	1.0 (0.4)	0.8 (0.6)	0.19 (0.04)	0.21 (0.05)
20.0	1.1 (0.1)	0.8 (0.2)	0.19 (0.02)	0.18 (0.03)
25.0	1.0 (0.7)	0.5 (0.3)	0.17 (0.04)	0.16 (0.50)
30.0	0.6 (0.2)	1.0 (1.0)	0.17 (0.03)	0.19 (0.04)

2 min. cGMP levels were not measured in zoospores stimulated by PA.

Phosphatidyl inositol phosphates and inositol phosphates

Zoospores contain small amounts of phospholipid with the R_F value reported for phosphatidyl inositol-4,5-bisphosphate (PIP₂). On the assumption that all phosphate pools in the mycelium were of the same specific activity, the absolute maximum concentration ranged between 20 and 50 pmol per 10^6 cells. There was no significant variation in these concentrations or consistent change in their pattern over the 10 min following pectate addition.

Concentrations of inositol phosphates in stimulated cells were also measured. The IP₃ concentrations measured by RIA increased from 4 pmol per 10^6 cells to 9 pmol per 10^6 cells over 8 min (Fig. 1). Levels in unstimulated cells also increased, and by 8 min equalled those in the stimulated cells (10 pmol per 10^6 cells). These small increases are in contrast to the 6-fold increases observed in mammalian cells, where IP₃ signals are known to be important (Palmer *et al.*, 1989). Assuming the zoospore to be a sphere with a mean diameter of 10 μ m, the concentration of IP₃ is of the order of 5 μ M, which is higher than in non-stimulated

Fig. 1. Inositol-1,4,5-trisphosphate in zoospores during the first 8 min following pectate addition. The values shown are the means of two separate experiments. Levels in stimulated cells shown as filled circles (●); in unstimulated cells as open circles (○).

Fig. 2. Diacylglycerols isolated from zoospores following stimulation with Tris-pectate and separated by GC. (a) Extract prepared from spores immediately prior to stimulation; (b) 5 s post stimulation; (c) 60 s post stimulation; (d) 5 min post stimulation; (e) non-stimulated population, 5 min after time set as zero. Data shown are actual tracings of GC separations, uncorrected for specific response factors of individual DAGs.

animal cells. There were small oscillations in IP₃ concentration in stimulated cells during the first minute, and between 2 and 5 min, the IP₃ concentrations in the differentiating cells were slightly above those in the controls.

When several inositol phosphates were measured from the radioactivity in each, calculating mass from the specific activity of *myo*-[³H]inositol in the growth medium, the values for the different inositol phosphates varied widely from one experiment to the next. The IP pool was always the largest, containing as a maximum, one nmol per 10⁶ cells. The inositol polyphosphates were present at between 0.5 and 10% of this level. The IP level increased slowly in stimulated cells, and at 8 min it was 50% above that observed prior to stimulation. The increases in IP₂ and IP₃ concentrations were smaller. The IP₄ level showed a 50% increase at 1 min, but by 90 s had returned to that in the control and did not change over the remainder of the period.

Diacylglycerols

At least 9 DAG isomers were distinguished in the total zoospore lipid extract (Fig. 2). While there was an increase in the total level of DAGs over a 5 min period in stimulated cells, differences between stimulated and control populations were not significant. There were small changes in the relative amounts of the DAG isomers which separated between 27.8 and 28.5 min, but these changes varied from one experiment to the next and no constant pattern was observed.

Addition of phorbol 12-myristate 13-acetate, which mimics the effects of DAGs in cellular metabolism in mammals (Berridge, 1984) did not induce zoospore differentiation at 1–10 μM. The addition of diolein or dipalmitin (45 μg ml⁻¹) was also without effect.

Phosphatidic acids

When ³²P-labelled phospholipids prepared from stimulated zoospores were separated by two dimensional TLC on silica gel plates, an increase in the amount of radioactivity was observed in a single spot on the chromatogram, located in the region where phosphatidic acids have been shown to run in these solvent systems. The amount of radioactivity in the putative PA increased rapidly in pectate-stimulated zoospores, reaching 1.8 times that of the non-stimulated cells within 10 s and between 2.5 to 3 times the initial concentration after 2 min (Fig. 3). The PA increase was also observed when cells were induced to differentiate by the addition of Sr²⁺ or by shaking (Fig. 3). In some experiments, the PA level continued to increase, until by 5 min it exceeded 4 times the level observed in the cell population prior to stimulus.

The value of the pre-stimulus level of PA was calculated to be 100 ± 20 pmol per 10⁶ cells based on the specific activity of the [³²P]phosphate in the growth medium.

Saponification of the material released palmitic acid and three unsaturated fatty acids; two were C:18 unsaturated fatty acids and the other a C:20 polyunsaturated fatty acid (Fig. 4), identified by retention times on GC and electron impact ion fragmentation patterns in the GC/MS. The saponification products also included phosphate and glycerol, but no other major peaks were evident. The material on the TLC plates

Fig. 3. Changes in phosphatidic acid concentrations in stimulated zoospores. Open circles (○), PA concentrations in cells stimulated with Tris-pectate; filled circles (●), those stimulated by addition of 3 mM-Sr²⁺; triangles (△), cells stimulated by shaking. The data shown in this figure are typical of those observed in five different experiments. There was some variation between experiments in the time taken for the PA concentration to reach a plateau. The level of phosphatidic acid in unstimulated cells in this experiment was 115 pmol per 10⁶ cells, calculated on the basis of specific activity of the phosphate in the medium on which the mycelia were grown.

Fig. 4. Profile of total products released from PA following saponification and derivatization. Zoospore lipids were separated by TLC and the material in the PA location eluted and saponified. The material was neutralized and after removal of HCl, was derivatized with BSTFA and separated by GLC. Peak 1 is phosphate; peak 2, glycerol; peak 3, palmitate; peak 4, two unresolved C:18 unsaturated fatty acids; and peak 5, an unsaturated C:20 fatty acid. The identity of each of the peaks was established by comparing their retention times with authentic standards and was confirmed in a separate GC/MS analysis. Materials eluting before phosphate and immediately following glycerol are from the derivatization reagents. Peaks at 45 and 49 min were not identified in GC/MS searches, but were not fatty acids.

therefore appears to contain at least two PAs, but nothing else.

When exogenous dipalmitoyl PA was applied to zoospore populations, cells differentiated with an efficiency almost equal to that observed when pectate was added as an inducer, although the onset of both encystment and germination were slightly slower (Fig. 5). Neither phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine nor choline chloride induced

Fig. 5. The kinetics of differentiation of *P. palmivora* zoospores induced by dipalmitoyl phosphatidic acid compared with that induced by Tris-pectate. The percentage of cysts formed is shown by circles, the percentage of germlings by triangles. Open symbols show populations to which 500 µg pectate ml⁻¹ has been added; filled symbols, populations to which 3 µM-PA had been added.

Fig. 6. The effect of the concentration of exogenous dipalmitoyl PA on *P. palmivora* zoospore encystment. Open circles (○), percentage of cysts formed 20 min after addition of PA; closed circles (●), percentage of germlings present 80 min after PA addition. The decrease in the percentage of cells completing differentiation at higher PA concentrations is the result of cell lysis.

differentiation. Maximum percentage differentiation was obtained at 2 µg ml⁻¹ (3 µM) PA (Fig. 6). Higher concentrations induced rapid cell lysis, so that percentage of the population forming cysts or germlings fell to almost zero at 10 µg ml⁻¹.

Interaction of PA and Ca²⁺

The lytic effect of exogenous PA was dependent on the concentration of Ca²⁺ in the suspension medium, which was normally 200 nM. When free Ca²⁺ was increased to 400 nM, more than 80% of the zoospore population formed cysts at PA concentrations of 10 µg ml⁻¹. Conversely, reduction of the free Ca²⁺ concentrations to 100 nM produced only 20% encystment at 5 µg ml⁻¹.

It had been shown previously that the calcium-channel blocker verapamil reduced the rate at which zoospores underwent differentiation following stimulation (Iser *et*

Table 2. *Changes in phosphatidic acid concentration under conditions reducing zoospore differentiation*

Tris-pectate, pH 6.2, was supplied at a concentration of 500 $\mu\text{g ml}^{-1}$; verapamil was added to a concentration of 30 μM . Tris-EGTA, pH 7.4, was added to a concentration of 100 μM . Phosphatidic acid was separated and quantified on the basis of specific activity of phosphate as described in Methods.

Treatment	PA level (% control) at 20 s	SE (n)	% Cysts at 10 min
Control	100	—	< 5
Pectate	240	37 (8)	92
Pectate + verapamil	133	8 (3)	72
Pectate + Tris-EGTA	154	28 (6)	10

SE, standard error.

al., 1989). In the presence of verapamil the onset of the production of PA was also significantly reduced (Table 2), with only a 33% increase above the control at 20 s, compared to a 140% increase in the absence of verapamil. A reduction in spore PA concentration of the same magnitude was observed when Tris/EGTA was used to reduce the external Ca^{2+} concentration (Table 2). Lowering of external Ca^{2+} had a much greater effect on encystment (which fell from 92% to 10%) than on PA production, while verapamil reduced PA to a greater degree than it reduced encystment, suggesting that any effect of Ca^{2+} is not expressed solely through an inhibition of PA production.

Source of phosphatidic acid

The impermeability of zoospores to most organic compounds (Penington *et al.*, 1989) and their general fragility when subjected to treatments such as electroporation or mild detergent treatment (B. R. Grant and J. R. Iser, unpublished results) made it impossible to introduce either exogenous ATP or phosphate to identify the source of the PA by pulse chase experiments. We were able to introduce some ^{32}P into spores by adding carrier-free [^{32}P]phosphate to sporangial suspensions immediately prior to zoospore release. This technique did not give reproducible labelling patterns, but it was sometimes possible to produce zoospores in which nucleotides, including ATP, were heavily labelled, while phospholipids contained little radioactivity. When these zoospores were induced to differentiate, no radiolabelled PA was detected.

In mammalian cells, PA is formed by several pathways. In signalling systems, PA is commonly produced by phosphorylation of DAG, produced by the action of phospholipase C (PLC) on phosphatidyl inositol phosphates (Berridge, 1987). PA appears as the

main product, accumulating after a few seconds (Mauco *et al.*, 1984). Transient elevations of diacylglycerols (DAG) are usually observed during the first 10 s after stimulation and precede the rise in PA (Kroll *et al.*, 1989). No such transient increases in DAGs were observed in this study, nor was there any increase in IP_3 , the other product of phospholipase C activity (Berridge, 1984). Studies with mammalian cells are generally carried out at 37 °C, while our experiments were carried out at 25 °C, so transient intermediates should be easier to observe if present. The increases in IP_3 (5 pmol per 10^6 cells) were far less than the increases in PA observed (more than 100 pmol per 10^6 cells). The measurements of the increase in all inositide phosphate pools are less reliable, but these could be as high as 500 pmol per 10^6 cells. This change is of the same order as that observed in the PA, and so the possibility that PA arises solely from PLC activity on PIP_2 cannot be excluded on this basis.

As no incorporation of ^{32}P into the PA pool was observed where endogenous ATP had been labelled, and only a small amount of label was present in the phospholipid pool, it is unlikely that synthesis of the additional PA occurred *de novo* from glycerol, through glycerol-3-phosphate and glycerol kinase activity (Brindley, 1985).

A third route to PA production is provided by the action of D-type phospholipases, producing PA directly from phospholipid hydrolysis (Loffelholz, 1989). Phosphatidyl choline (Cabot *et al.*, 1988; Martin, 1988) and phosphatidyl inositol (Cockcroft, 1984) have been reported as preferred substrates for these D-type phospholipases in mammalian cells. As we have detected phospholipase D activity in extracts of these spores, but no phospholipase C activity, our results are consistent with PA formation through this pathway.

Discussion

The induction of encystment in *P. palmivora* zoospores is very rapid, and in the presence of pectate is normally followed by germination. The earliest response previously recorded, an increase in the rate of Ca^{2+} exchange, had a $t_{1/2}$ of 30 s or less (Irving *et al.*, 1984). Although many intracellular messengers may be involved in the regulation of spore germination, any compound involved in the direct transmission of the external signal to encyst must respond within a time scale of seconds, which is also the time scale within which spores become committed to germination (Paktitis *et al.*, 1986).

Substantial increases in the size of the PA pool were observed in all conditions under which zoospore differentiation was induced and took place during this period.

Analysis of the acyl components of the PA suggested that there was more than one species of PA in the pool, and that palmitate formed one of the acyl groups. Exogenous dipalmitoyl PA induced differentiation very effectively, but it is not clear whether there is a specific acyl group requirement for the induction process. Other phospholipids did not induce spore differentiation, suggesting a specific PA requirement.

The role of PA as a second messenger in the processes of cell differentiation and secretion has recently come under intensive scrutiny in mammalian systems, particularly with the realization that it can arise directly from phospholipase D (PLD) action, as well as indirectly from the phosphorylation of diacylglycerols produced by phospholipase C. Increases in PA levels associated with stimulus-coupled secretion or the resumption of mitotic division have been observed in a wide range of animal cell types. The role of PA and PLD has been reviewed by Exton (1990) and by Shulka & Halenda (1990). A preliminary report has also appeared, suggesting that PA acts as a second messenger in the signals generated in the pulvini of plants (Coté *et al.*, 1989). So far as we are aware, ours is the first report of an increase in PA in cells of a lower eukaryote under conditions where it might function as a second messenger.

Our results are consistent with PA production by way of a D-type phospholipase, operating independently of phospholipase C and possibly utilizing phosphatidyl choline as a substrate. The kinetics of PA production suggest that PLD activation may result directly from the external stimulus which induces differentiation. However, it is emphasized that the data do not totally exclude the possibility of a PLC-generated signal coupled with a very rapid kinase reaction.

The role of cyclic nucleotides as second messengers during differentiation in these cells remains unclear. Although both cGMP and cAMP concentrations increased within 20 s in *P. palmivora* spores, the increases were relatively small. The larger, transient increase in cAMP at around 4 min after pectin addition comes too late to be involved in transmission of the external stimulus, but small increases occur over the time that PA begins to increase. It is possible that the magnitude of changes in cyclic nucleotides may be an underestimate, resulting from artificially high levels in the controls, induced by handling stresses. Zoospores do differentiate in the absence of pectin addition, although more slowly and asynchronously. The level of both cyclic nucleotides observed in cells prior to stimulation is at the upper end of the range reported by Goldberg *et al.* (1973) for a range of unstimulated mammalian cells.

Exogenous cAMP and cGMP both induced *P. palmivora* spore differentiation and the associated Ca^{2+} efflux (Irving *et al.*, 1984), but only at mM concentrations,

as might be expected from the known low permeability of nucleotides. There is no evidence for cyclic nucleotide receptors on the *P. palmivora* spore surface, such as have been demonstrated on the surface of *Dictyostelium discoideum* (Schaap & Wang, 1985).

Although cyclic nucleotides have been reported to function as second messengers in spores of the chytrid fungus *Blastocladiella emersonii*, which differentiate in a similar manner to those of *Phytophthora*, the evidence is not particularly strong. cAMP-binding proteins have been isolated, and cAMP-dependent protein phosphorylation has been observed in these spores (Brochetto-Braga *et al.*, 1982; Gomes *et al.*, 1983), as have elevations in internal cAMP concentrations during germination (Vale *et al.*, 1976; Gomes *et al.*, 1980). However, the kinetics of these increases make it unlikely that cAMP was acting as the second messenger during the initiation of encystment in *B. emersonii* spores any more than in *P. palmivora*. Moreover, when *B. emersonii* zoospores were induced to differentiate by the addition of K^+ , no increase in cAMP level was observed and the addition of permeable N^6 - O^2 -dibutyryl cyclic nucleotides failed to induce differentiation (Silverman & Epstein, 1975). We conclude that there is no convincing evidence for cyclic nucleotide involvement in the early stages of differentiation in either species.

Inositol phosphate(s) and diacylglycerols, which are products of phospholipase-C-mediated catabolism of phosphatidyl 4,5-inositol-bisphosphate, are clearly present in *P. palmivora* zoospores. Failure to observe the true increase in IP_3 in these experiments could be the result of artificially elevated levels in the unstimulated cells, as for the cyclic nucleotides. However, the small changes in IP_3 levels contrast with those observed in PA, and suggest that this pathway has no role in the transmission of the external signals which induce the encystment phase of the differentiation process.

In *Phytophthora* spores, differentiation is critically dependent upon external Ca^{2+} concentration, and PA production via PLD requires extracellular Ca^{2+} . The neutral D-type phospholipase from mammalian neutrophils requires Ca^{2+} concentrations in the nM to μM range (Balsinde *et al.*, 1989; Huang *et al.*, 1991) which is the same range of Ca^{2+} concentrations as that required for zoospore differentiation (Griffith *et al.*, 1988). If it is established that a PLD signalling system alone initiates encystment in these spores, they offer a unique opportunity to study this mechanism free from the problems found in animal cells, where both PLC and PLD signal generators are present together.

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