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A role for calcium in appressorium induction in *Phytophthora palmivora*

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Abstract

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Appressorium formation *in vitro* by *Phytophthora palmivora* was strongly influenced by Ca^{2+} ions: treatment of zoospores with an inhibitor of stretch-activated ion channels (Gd^{3+}), an inorganic Ca^{2+} channel blocker (Nd^{3+}), or EGTA inhibited appressorium formation. The Ca^{2+} ionophore A23187 had a positive effect on this process which points to an important role of increased Ca^{2+} levels for appressorium formation. The latter is known to start about 60 min after incubation of zoospores. This early preparatory phase corresponds to the time period found to be sensitive to an inhibition of Ca^{2+} channels by Nd^{3+}. The calmodulin antagonist TFP inhibited appressorium formation, pointing to an involvement of the Ca^{2+}-calmodulin complex in this process.

Our experiments do not point to a role of cAMP in appressorium induction. Exogenously applied cAMP and some compounds (NaF, 2-deoxyadenosine and IBMX) known to elevate endogenous levels of cAMP did not induce appressorium formation.

Key words: Infection structure, Calcium, Calmodulin, cAMP, *Phytophthora palmivora*.

Introduction

Phytophthora palmivora causes diseases in a broad range of economically important crops in warmer regions of the world (Chee 1974, Erwin & Ribeiro 1996). The infective capability on aerial parts of its hosts depends on the production of a series of infection structures including germ-tubes, appressoria and penetration hyphae (Feuerstein & Hohl 1986). The appressorium is a specialized infection structure produced by many pathogenic fungi, which provides the germ-tube with the capacity to strongly adhere to the host surface in preparation for subsequent invasion (Emmett & Parbery 1975).

The ability to induce appressorium formation *in vitro* has faciliated the study of environmental signals affecting this process in *P. palmivora* (Bircher & Hohl 1997a). We found that appressorium induction was influenced by contact with a surface, substrate hydrophobicity and the nutrient status of the surrounding medium.

How these signals, once perceived, are translated into specific intracellular responses that lead to infection structure differentiation, is unknown. The mechanisms of recognition of environmental stimuli and signal transduction are well understood in animal systems and appear to be fundamentally similar in all eukaryotes (Gadd 1995). A number of molecules have been identified as important intracellular mediators of extracellular signals, such as the cyclic nucleotide adenosine 3′,5′-cyclic monophosphate (cAMP) or Ca^{2+} (Laychock 1989). These second messengers are also important regulators of development in fungi, as shown by direct and indirect evidence from a variety of experimental systems (Jackson & Heath 1993, Gadd 1995).

The aim of the present study was to determine the role of cAMP and Ca^{2+} in appressorium induction in *P. palmivora*. To achieve this, we used substances that act on the key enzymes of cAMP metabolism causing an accumulation of cAMP in the cells (Kalderon, Dobbs & Greenberg 1980, Lee & Dean 1993). The role of intracellular Ca^{2+} levels was studied by blocking Ca^{2+} channels, chelating Ca^{2+} with EGTA, or by using a Ca^{2+} ionophore.

Materials and methods

Growth of the pathogen and production of zoospores

Phytophthora palmivora (Butler) Butler strain P113 was maintained as described by Bircher & Hohl (1997a, 1997b). For zoospore production sporangia were transferred into 15 ml of sterile distilled water and incubated at 4 °C for 15 min, and for an additional 20 min at 25 °C on an orbital shaker rotating at about 50 rpm to induce emergence of zoospores.

After filtration through a 20 µm nylon mesh (Nybolt P20, Schweizerische Gazefabrik AG) to separate the zoospores from the sporangial cases the concentration of the zoospores was determined using a haemocytometer. The zoospore suspension was diluted with pea broth (Hohl & Balsiger 1986, Bircher & Hohl 1997a) and distilled water to a final concentration of 5×10^4 zoospores ml^{-1}, and used immediately as a 5% (v/v) pea broth solution. Addition of pea broth did not induce rapid encystment of zoospores.

Test substances

The following substances were used: 2-deoxyadenosine, cAMP and the calcium ionophore A23187 purchased from Sigma (Buchs, Switzerland), EGTA (ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid) from Serva (Heidelberg, Germany), IBMX (3-isobutyl-1-methylxanthine) and neodymium chloride ($NdCl_3$) from Fluka (Buchs, Switzerland), gadolinium chloride ($GdCl_3$) from Heraeus (Karlsruhe, Germany), and sodium fluoride (NaF) from Merck (Dietikon, Switzerland). EGTA was dissolved in distilled water and titrated to pH 6.5 with a solution of 2 N NaOH. The calcium ionophore A23187 was dissolved in a small amount of ethanol before it was added to the incubation medium. The final ethanol concentration in the assay was 0.5% (v/v). All other chemicals were dissolved in distilled water.

Appressorium formation assay

To study the influence of different substances on appressorium formation *in vitro* two substrates were chosen: glass coverslips (Menzelgläser, Merck, Dietikon, Switzerland), and 35 mm diameter polystyrene Petri dishes (Greiner GmbH, Nürtingen, Germany). To provide topographically structured surfaces polystyrene Petri dishes were scratched repeatedly with a brass brush to ensure that most adhering germlings contacted at least one scratch during the incubation period (Bircher & Hohl, 1997a).

The test substances were added to zoospores diluted in 5% (v/v) pea broth. After incubation for 3 h at 25 °C in darkness appressorium formation was immediately checked with an inverted microscope

(Nikon, TMS-F) equiped with a 20× Ph 2 DL objective (Nikon). The experiments were performed as triplicates. In each of these independent experiments, at least 3×200 germlings were analysed.

Interference contrast observations were made on an Axiophot (Zeiss) microscope equipped with a Plan-NEOFLUAR 40 (Ph 2) objective.

Results

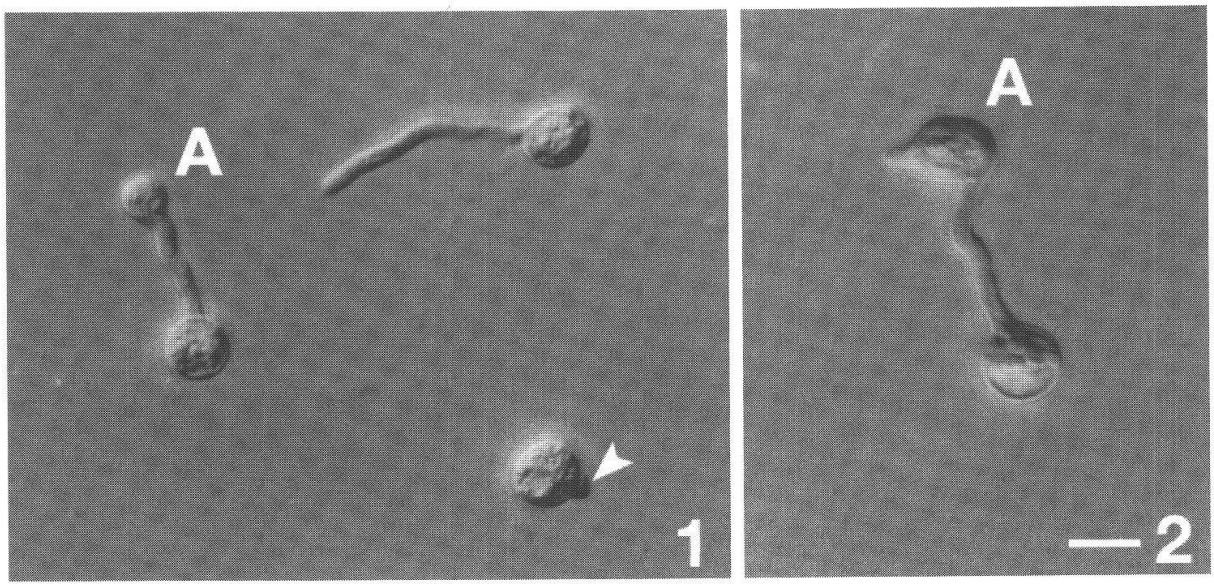
Germination and differentiation

In untreated controls, zoospores of *P. palmivora* adhered to a polystyrene surface, formed a cyst wall and germinated by producing mostly single germ-tubes (Fig. 1). After appressorium induction and maturation, finger-like projections (Fig. 2) and/or one or several vesicles emerged from these appressoria.

Significantly more germlings formed appressoria on a scratched surface than on a smooth surface ($t=-4.48$, $DF=16$, $\alpha=0.004$) after 3 h of incubation in 5% (v/v) pea broth (Table 1).

Effects of gadolinium (Gd^{3+}) on development

Addition of 150 µM $GdCl_3$ to the incubation medium [5% (v/v) pea broth] inhibited appressorium formation of germlings adhering to smooth and scratched polystyrene completely (Table 1). To determine whether the effect of $GdCl_3$ depended on Gd^{3+} or Cl^-, the 150 µM $GdCl_3$ was replaced with either 500 µM NaCl or $CaCl_2$. We found that appressorium formation was not affected in these controls, neither on smooth nor on scratched polystyrene.



Figs. 1, 2. Developmental stages of *Phytophthora palmivora* on smooth polystyrene. Fig. 1. Cyst with emerging germ-tube (arrow head), germling with elongating germ-tube and germling with maturing appressorium (A). Fig. 2. Mature appressorium (A) with emerging hypha. Bar represents 10 µm for both figures.

Table 1. Influence of gadolinium (Gd^{3+}), an inhibitor of stretch-activated ion channels, on appressorium formation of *Phytophthora palmivora* on smooth or scratched polystyrene.

Test substance	Concn (μM)	% Adhering germlings with appressoria on polystyrene [a]	
		Smooth surface	Scratched surface
$GdCl_3$	150	0.1 ± 0.2	1.6 ± 1.5
NaCl	500	87.4 ± 4.6	93.5 ± 3.4
$CaCl_2$	500	83.5 ± 4.4	93.8 ± 2.2
Control:			
5% (v/v) pea broth		82.7 ± 3.8	90.2 ± 3.3

[a] Germlings were incubated for 3 h at 25 °C. At least 200 germlings were analysed per test substance in each of three replicates. Three independent experiments were performed. Values represent mean and S. D. ($n=9$).

Effects of a Ca^{2+} channel blocker (Nd^{3+}), EGTA, and a Ca^{2+} ionophore (A23187) on differentiation

On smooth polystyrene, EGTA treatment led to a dose-dependent reduction of the frequency of appressorium formation (Fig. 3) resulting in about 20% of adhering germlings that form appressoria at 1 mM EGTA. Similar results were obtained with the inorganic Ca^{2+} channel blocker Nd^{3+} which significantly reduced appressorium formation of adhering germlings at micromolar levels (Fig. 4). At 100 μM Nd^{3+} only low frequencies of appressoria were induced, and no hyphae and/or vesicles emerged from the appressoria.

The Ca^{2+} ionophore A23187 had a slight but significant positive effect on appressorium formation of germlings adhering to smooth polystyrene (Fig. 5). At 1 μM and 10 μM the ionophore induced significantly more adhering germlings to form appressoria compared to germlings in the corresponding control containing 0.5% (v/v) ethanol ($t=-3.403$, $DF=16$, $\alpha=0.0036$ for 1 μM A23187; $t=-8.881$, $DF=16$, $\alpha=0.0001$ for 10 μM A23187).

While Nd^{3+} (100 μM) reduced appressorium formation on scratched and on smooth polystyrene to about 10%, EGTA (1 mM) inhibited appressorium formation on smooth polystyrene only, and A23187 did not increase infection structure formation on scratched surfaces (Table 2).

Time period of Ca^{2+} channel activity needed for appressorium induction

In order to determine the time period in development, during which Ca^{2+} channel activity is needed for appressorium induction, zoospores were incubated in 5% (v/v) pea broth for 3 h. At various times during this incubation period Ca^{2+} channels were blocked by adding Nd^{3+} at 100 μM to the incubation medium, where it remained until appressorium formation was determined (Table 3). Addition of Nd^{3+} during the first 0–30 min of incubation totally inhibited appressorium formation, while the inhibitory effect was progressively less pronounced if Nd^{3+} was added after 60 min or later.

To test whether or not the inhibitory effect of Nd^{3+} at 100 μM is reversible, zoospores were incubated in 5% (v/v) pea broth containing the inhibitor. At various times, the Nd^{3+} containing incubation medium was replaced by a 5% (v/v) pea broth solution. Appressorium for-

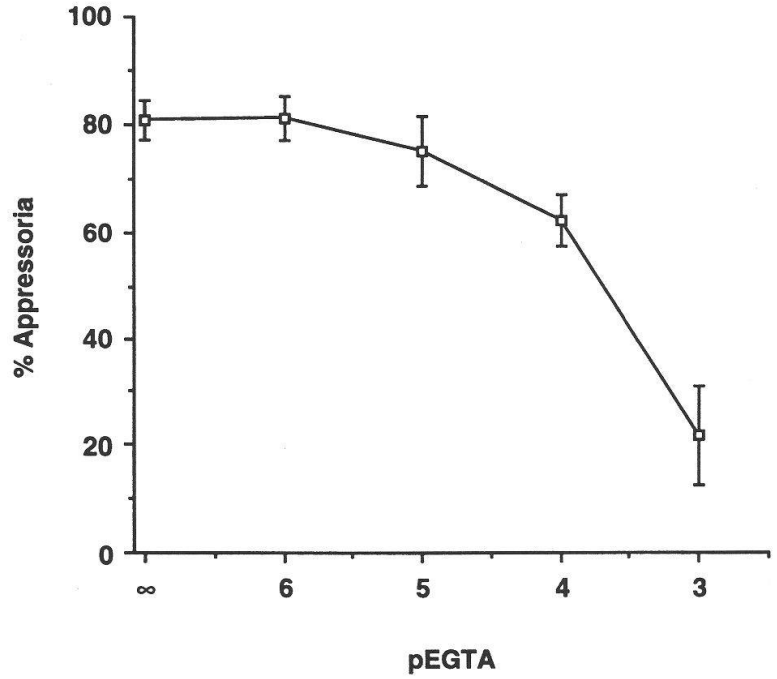


Fig. 3. The effect of EGTA on appressorium formation on smooth polystyrene. Germlings were incubated for 3 h at 25 °C. At least 200 germlings were analysed per treatment in each of three replicates. Three independent experiments were performed. Values represent mean and S.D. ($n=9$). pEGTA = –log[EGTA].

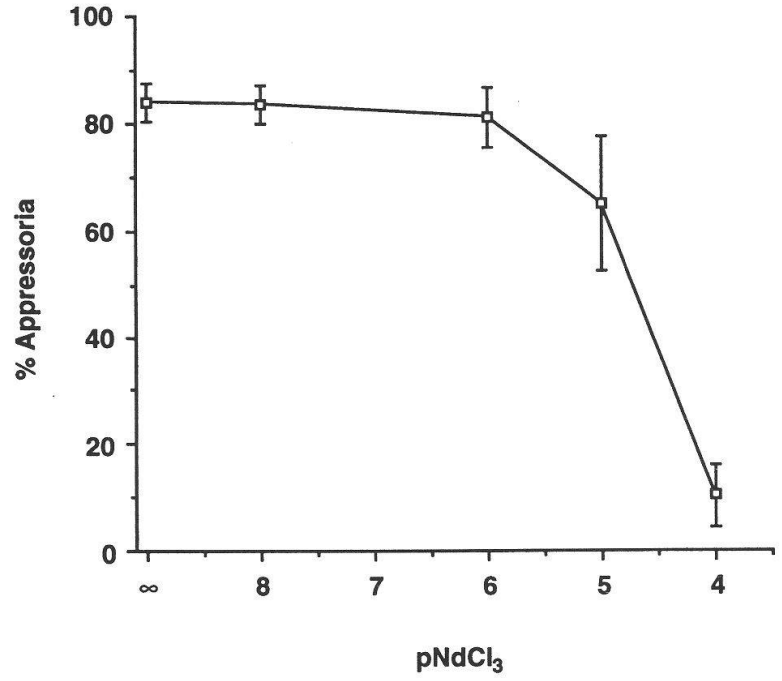


Fig. 4. The effect of the Ca^{2+} channel blocker neodymium (Nd^{3+}) on appressorium formation on smooth polystyrene. Germlings were incubated for 3 h at 25 °C. At least 200 germlings were analysed per treatment in each of three replicates. Three independent experiments were performed. Values represent mean and S.D. ($n=9$). $pNdCl_3 = -\log[NdCl_3]$.

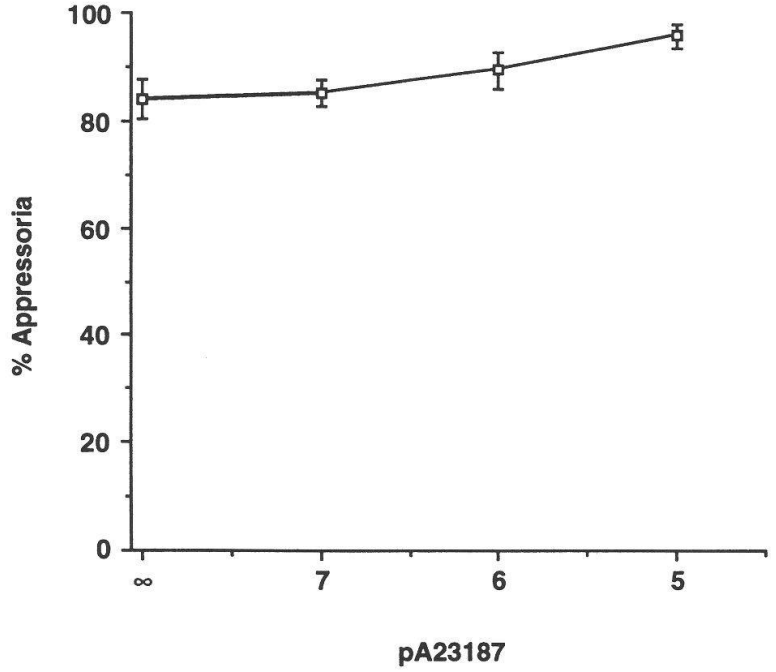


Fig. 5. The effect of the Ca^{2+} ionophore A23187 on appressorium formation on smooth polystyrene. Germlings were incubated for 3 h at 25 °C. At least 200 germlings were analysed per treatment in each of three replicates. Three independent experiments were performed. Values represent mean and S. D. ($n=9$). pA23187 = –log[A23187].

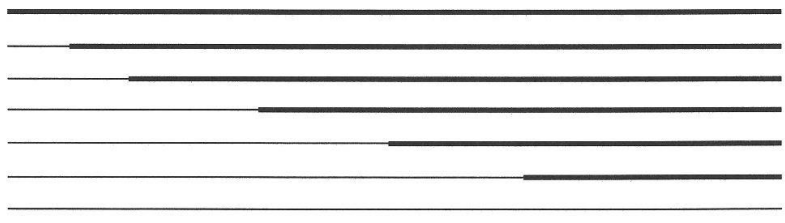
Table 2. Influence of the Ca^{2+} channel blocker neodymium (Nd^{3+}), EGTA, and the Ca^{2+} ionophore A23187 on appressorium formation of *Phytophthora palmivora* on smooth or scratched polystyrene.

Test substance [a]	% Adhering germlings with appressoria on polystyrene [b]	
	Smooth surface	Scratched surface
$NdCl_3$ (100 µM)	10.3 ± 5.7	9.9 ± 7.2
EGTA (1 mM)	21.8 ± 9.2	74.6 ± 4.2
A23187 (10 µM)	95.9 ± 2.2	95.6 ± 1.7
Controls:		
5% (v/v) pea broth	83.8 ± 3.6	92.1 ± 2.2
0.5% (v/v) ethanol	84.0 ± 3.4	91.4 ± 2.6

[a] $NdCl_3$ and EGTA were dissolved in 5% (v/v) pea broth. A23187 was dissolved in a small amount of ethanol, before it was added to the incubation medium. The final ethanol concentration was 0.5% (v/v).
[b] Germlings were incubated for 3 h at 25 °C. At least 200 germlings were analysed per test substance in each of three replicates. Three independent experiments were performed. Values represent mean and S. D. ($n=9$).

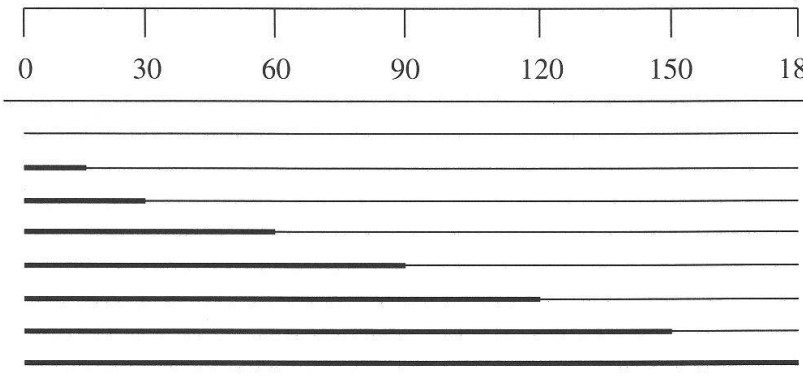
mation was determined after 3 h of incubation (Table 4). The results show that presence of Nd^{3+} during the first 0–60 min of incubation did not repress appressorium formation, while presence for the first 90 min or longer significantly repressed appressorium formation ($t=10.911$, $DF=16$, $\alpha=0.0001$ for 60 min versus 90 min).

Table 3. Influence of the Ca^{2+} channel blocker neodymium (Nd^{3+}) added after various times of incubation on appressorium formation on smooth polystyrene in *Phytophthora palmivora.* The presence of neodymium is indicated by the heavy lines.

Incubation time (min) * 0 30 60 90 120 150 180	% Adhering germlings with appressoria [a]
	0.7 ± 0.7
	0.7 ± 0.7
	1.1 ± 0.9
	8.5 ± 5.0
	41.3 ± 6.8
	65.9 ± 7.3
	84.2 ± 4.0

[a] Germlings were incubated for 3 h at 25 °C. $NdCl_3$ was added to the incubation medium [5% (v/v) pea broth]. At least 200 germlings were analysed per incubation time in each of three replicates. Three independent experiments were performed. Values represent mean and S. D. ($n = 9$).
* Appressorium formation starts at this point (Bircher & Hohl, 1997a).

Table 4. Influence of neodymium (Ca^{2+} channel blocker) removal after various times of incubation on appressorium formation on smooth polystyrene in *Phytophthora palmivora.* The presence of neodymium is indicated by the heavy lines.

Incubation time (min) 0 30 60 90 120 150 180	% Adhering germlings with appressoria [a]
	85.2 ± 2.6
	85.1 ± 5.0
	86.4 ± 4.0
	87.7 ± 2.3
	66.9 ± 5.2
	67.8 ± 8.1
	59.1 ± 4.6
	7.6 ± 6.7

[a] Germlings were incubated for 3 h at 25 °C. The neodymium chloride ($NdCl_3$) containing inubation medium was replaced by a 5% (v/v) pea broth solution. At least 200 germlings were analysed per incubation time in each of three replicates. Three independent experiments were performed. Values represent mean and S. D. ($n = 9$).

Effects of a calmodulin antagonist on appressorium induction

Germlings were also treated with the calmodulin antagonist trifluoperazine (TFP). The results are presented in Fig. 6. The antagonist inhibited appressorium formation of adhering germlings in a dose-dependent manner at concentrations between 1–5 µM resulting in 14.9 ± 5.6% of germlings that form appressoria at 5 µM TFP.

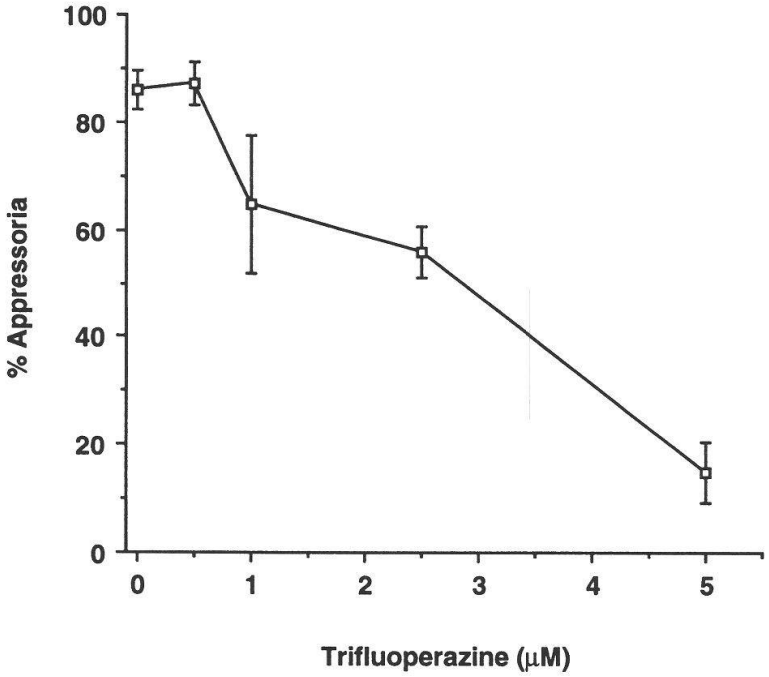


Fig. 6. The effect of the calmodulin antagonist trifluoperazine (TFP) on appressorium formation on smooth polystyrene. Germlings were incubated for 3 h at 25 °C. At least 200 germlings were analysed per treatment in each of three replicates. Three independent experiments were performed. Values represent mean and S. D. ($n = 9$).

Table 5. Influence of exgenous cAMP and chemicals which are known promoters of increased levels of endogenous cAMP, on appressorium formation in *Phytophthora palmivora* on glass.

Test substance [a]	Concn (mM)	% Adhering germlings with appressoria [b]
cAMP	0.001	2.8 ± 1.6
	10	1.3 ± 2.1
NaF	1	2.6 ± 1.8
	5	4.1 ± 1.9
2-Deoxyadenosine	5	1.6 ± 0.8
	10	0.7 ± 0.6
IBMX	0.5	5.5 ± 2.7
	1	3.7 ± 1.7
Control:		
5% (v/v) pea broth		0.9 ± 0.5

[a] NaF and 2-deoxyadenosine are stimulators of adenylate cyclase (cAMP synthesis), 3-isobutyl-1-methylxanthine (IBMX) inhibits phosphodiesterase activity (cAMP breakdown).
[b] Germlings were incubated for 3 h at 25 °C. At least 200 germlings were analysed per test substance in each of three replicates. Three independent experiments were performed. Values represent mean and S. D. ($n = 9$).

Effects of exogenous and endogenous cAMP on development

We also tested whether or not cAMP had an enhancing effect on appressorium formation on glass, a hydrophilic substrate which does not lead to appressorium formation (Table 5). In the presence of exogenous cAMP, or chemicals known to increase the level of endogenous cAMP in some systems (NaF, 2-deoxyadenosine and IBMX), appressorium formation was only very slightly induced compared to controls incubated in 5% (v/v) pea broth.

Discussion

The experiments performed in this study were designed to examine potential involvements of cAMP, Ca^{2+} and calmodulin in appressorium induction in the oomycete *P. palmivora*. The results obtained are consistent with the idea developed for some other fungi (Magalhaes et al. 1991, Warwar & Dickman 1996) that this process is strongly influenced by intracellular Ca^{2+} levels. Support for this has been obtained by several lines of evidence: (1) entry of Ca^{2+} into the cytoplasm of fungal cells is thought to occur primarily at the plasma membrane, where stretch-activated channels permeable for Ca^{2+} (and other ions) have been identified and characterized by patch clamping protoplasts (Gustin et al. 1988, Zhou et al. 1991; Garrill, Lew & Heath 1992). A specific inhibitor of stretch-activated channels (Gd^{3+}) described by these authors and used in our study substantially repressed appressorium formation in *P. palmivora*. (2) The putative Ca^{2+} channel blocker Nd^{3+} (Wayne 1985) showed an inhibitory effect on appressorium initiation in our system, and (3) the same was found for low external Ca^{2+} concentrations achieved by the addition of EGTA. (4) Our studies on restricting Ca^{2+} entry into the cell were complemented by an experiment aimed at increasing the intracellular free Ca^{2+} concentration through the use of the ionophore A23178, which is known to elevate the rate of calcium uptake in *P. palmivora* (Irving, Griffith & Grant 1984). Its dose-dependent inducing effect on the frequency of germlings forming appressoria points to an important role of increased Ca^{2+} levels for this process.

Appressorium formation in *P. palmivora* starts about 60 min after incubation of zoospores on polystyrene (Bircher & Hohl 1997a). This early preparatory phase corresponds to the time period found to be sensitive to an inhibition of Ca^{2+} channels by Nd^{3+}. Addition of Nd^{3+} during the first 0–30 min of incubation and its presence for the remaining incubation period totally inhibited appressorium formation, while a significant induction of appressoria was noted if Nd^{3+} was added after 60 min.

Our results also show that the inhibitory effect of blocking Ca^{2+} channels by Nd^{3+} is reversible. Removal of the inhibitor after 150 min of incubation was sufficient to induce high frequencies of appressoria within 30 min of incubation in the absence of Nd^{3+}. This result supports the idea of Magalhaes et al. (1991) that an initial Ca^{2+} deprivation, mimicked in our system by blocking Ca^{2+} channels, does not prevent appressorium formation if external Ca^{2+} becomes available. This is a property that appears advantageous for a pathogen which may experience low levels of Ca^{2+} while growing on the surface of a host plant.

In animals, Ca^{2+} acts as second messenger in many stimulus-response systems. After activation by a primary stimulus, the intracellular concentration of free Ca^{2+} rises from 0.1 µM to 1–10 µM (Kretsinger 1981). At this concentration, Ca^{2+} binds to Ca^{2+}-binding proteins such as calmodulin, which in turn may activate essential processes in the cell. Calmodulin is present in a variety of fungi (Gadd 1995), including *Phytophthora* (Gubler et al. 1990, Pieterse, Verbahel & Spaans 1993). An involvement of the Ca^{2+}-calmodulin complex in appressorium fomation in *P. palmivora* is supported by the inhibitory effect of the putative calmodulin antagonist TFP. A more precise evaluation of the role of calmodulin in appressorium

induction in *Phytophthora* might be done by inhibiting the expression of the calmodulin gene(s), one of which has recently been cloned in *P. infestans* (Pieterse, Verbahel & Spaans 1993).

Interestingly, our results suggest that the induction of appressoria on smooth substrates requires higher external Ca^{2+} levels than the induction on surfaces that provide inducive topographical signals, since at low external Ca^{2+} levels more appressoria were induced on scratched than on smooth surfaces. A possible explanation for this situation might be that the rate of Ca^{2+} influx is increased after the germ-tube tip encounters an inducive topographical signal. The latter may open Ca^{2+} permeable stretch-activated channels in the stretched part of the plasma membrane, which would result in a higher rate of Ca^{2+} influx. This notion is further supported by the observation that inhibition of stretch-activated channels by Gd^{3+} totally repressed appressorium formation on both, scratched and smooth surfaces, and by the result that on smooth substrates the Ca^{2+} ionophore A23187 induced higher frequencies of appressoria than in controls.

Exogenously applied cAMP did not induce appressorium formation on glass, a substrate on which germ-tubes do not develop appressoria (Bircher & Hohl 1997a). It cannot be excluded, however, that exogenously applied cAMP did not enter the cells, since the cell wall and the plasma membrane are likely to be relatively impermeable for cAMP (Lee & Dean 1993). Furthermore, appressorium formation was also not induced by compounds known to elevate endogenous levels of cAMP (Kalderon, Dobbs & Greenberg 1980; Lee & Dean 1993), while some of these substances induced high frequencies of appressoria in *Magnaporthe grisea* (Lee & Dean 1993) and *Uromyces phaseoli* (Hoch & Staples 1984). Taken together, our results do not point to a role for cAMP in infection structure formation in *P. palmivora*.

In summary, our data support the concept that elevated levels of Ca^{2+} ions are important in early stages of infection structure formation in *P. palmivora*. They are consistent with the idea of Hardham (1992) that recognition of an inducive signal for appressorium formation leads to the opening of calcium channels and, therefore, to a rise in cytosolic free Ca^{2+}. The latter is likely to affect cytoskeletal elements and/or secretion, thereby conceivably initiating appressorium formation.

Zusammenfassung

Die Appressorienbildung bei *Phytophthora palmivora* wird stark durch Kalzium Ionen beeinflußt. Die Gegenwart eines Inhibitors von dehnungs-aktivierten Ionenkanälen (Gd^{3+}), eines anorganischen Kalziumkanal-Hemmstoffes (Nd^{3+}) und des Kalzium-bindenden EGTA hemmt die Appressorienbildung. Dagegen zeigt die Zugabe des Kalziumionophors A23187 einen positiven Effekt auf diesen Prozeß. Dies deutet darauf hin, daß eine erhöhte Konzentration an intrazellulärem Kalzium die Appressorienbildung beeinflußt. Letztere beginnt bei *P. palmivora* etwa eine Stunde nach der Inkubation von Zoosporen. Diese frühe Vorbereitungsphase entspricht der für den Kalziumkanal-Hemmstoff Nd^{3+} empfindlichen Zeitperiode. Der Calmodulin-Antagonist TFP hemmt die Appressorienbildung ebenfalls, was darauf hindeutet, daß der Kalzium-Calmodulin Komplex bei der Bildung von Infektionsstrukturen eine Rolle spielt. Hingegen finden wir keine Hinweise für eine Beteiligung von cAMP an diesem Prozeß. Exogen zugegebenes cAMP sowie Stoffe (NaF, 2-Deoxyadenosin und IBMX), welche dafür bekannt sind, den intrazellulären Gehalt an cAMP zu erhöhen, induzieren keine Appressorien.

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