Second messenger involvement in differentiation of the entomopathogenic fungus *Metarhizium anisopliae*

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(Received 22 December 1989; revised 1 May 1990; accepted 4 May 1990)

The conidial germling of *Metarhizium anisopliae* produces an appressorium upon contact with a hard hydrophobic surface. We have conducted an investigation into how this entomopathogen mediates intracellularly the inductive signal to shift from polarized germ-tube growth to non-polarized appressorial growth. During sporulation, conidia accumulated $^{45}\text{Ca}^{2+}$ but there was no evidence for a gradient of Ca^{2+} in the spore which could establish the initial polarity. Calmodulin, however, was localized at the poles of the conidia, near the site of germ-tube emergence. Exposing conidia to Ca^{2+} deprivation or calmodulin antagonists inhibited germination and polar growth. Disruption of Ca^{2+} gradients by ionophoresis did not prevent germination but caused the multiple emergence of branched germ-tubes from conidia. These findings indicate that Ca^{2+} plays a fundamental role in establishing the dominance of apical growth. Although an external source of Ca^{2+} is not required for appressorium formation, germlings producing appressoria took up $^{45}\text{Ca}^{2+}$ when available. The $^{45}\text{Ca}^{2+}$ accumulated in the cell wall, plasma membrane and organelles suggesting that these may function as Ca^{2+} stores for Ca^{2+} -stimulated exocytosis of cell-wall materials. Mitochondria and vacuoles sequestered $^{45}\text{Ca}^{2+}$ indicating that they play a role in maintaining low cytoplasmic concentrations of $^{45}\text{Ca}^{2+}$ consistent with the reorganization of the cytoskeleton required for appressorial growth. Several Ca^{2+} -binding proteins in appressoria may provide an energy-independent component of Ca^{2+} buffering in the cytoplasm. The results indicate that the apical Ca^{2+} gradient is disrupted during differentiation and subsequent differential Ca^{2+} redistribution in the cell enlargement zone coincides with germ-tube swelling. cAMP may also be involved by potentiating the effects of small changes in Ca^{2+} concentration and stimulating exocytosis of mucus components required for adhesion, which is a prerequisite for differentiation.

Introduction

Fungal hyphae are highly polarized structures that extend by the fusion of secretory vesicles with the plasma membrane of the hyphal apex. The rapidly extending tip is protected from the effects of the internal hydrostatic pressure which supplies the driving force for forward expansion by a thin cell wall supported by the actin cytoskeleton. During extension, new cell wall is displaced to the side walls and becomes hardened (secondary wall formation) by chemical cross-linking of wall components forming a structure rigid enough to with-

stand turgor pressure (Jackson & Heath, 1989; Wessels, 1986). The polarized mode of extension is manifested in many physiological features including the distribution of cytoskeletal elements and organelles (Jackson & Heath, 1989; McKerracher & Heath, 1987), the chemical composition and distribution of ions (Wessels, 1986) and consequently the electrical characteristics of the organism (Takeuchi *et al.*, 1988). A model has been proposed to account for polarity in tip-growing systems in which Ca^{2+} plays a major role in the control of both vesicle fusion and the plasticity of the tip (Picton & Steer, 1982).

By contrast to tip-growth, the branching process and other differentiation events occurring in fungi are poorly understood. Fungi respond morphologically to a very wide array of physical and chemical signals including touch, light, pheromones and nutrients. The mode of signal reception is poorly understood except in *Dictyostelium* where a complex transmembrane system for cAMP has been elucidated (Gerisch, 1987). Germ-tubes of the entomopathogenic fungus *Metarhizium anisopliae* are

Abbreviations: Br-A23187, 4-bromo-A23187; CaM, calmodulin; CTC, chlorotetracycline; DAPI, 4',6-diamidino-2-phenylindole; DCCD, *N,N'*-dicyclohexylcarbodiimide; H-8, *N*-(2-methylamino)-ethyl-5-isoquinolinesulphonamide dihydrochloride; IBTX, 3-isobutyl-1-methylxanthine; Sp-(cAMPS), adenosine 3':5'-monophosphothiate, cyclic Sp-isomer; W-5, *N*-(6-aminoethyl)-1-naphthalene-sulphonamide; YEM, yeast extract medium.

induced by contact with an appropriate surface to form terminal swellings (appressoria) in which a reduction in apical elongation is accompanied by an apparent collapse of polarity. Appressoria play key structural and functional roles in the entomopathogenicity of *Metarhizium* (St Leger *et al.*, 1987, 1989*a, b*; Zacharuk, 1970). Appressoria are a major site of adhesion of the germling to the host and may provide a fulcrum for mechanical pressure exerted by infection pegs. As active producers of cuticle-degrading enzymes they play a crucial, if not exclusive role, in establishing a nutritional relationship with the host (Goettel *et al.*, 1989; St Leger *et al.*, 1987, 1989*b*). In contrast to germination, differentiation of infection structures by *Metarhizium* occurs only in response to specific host-related stimuli (a hard hydrophobic surface and low levels of complex nitrogenous nutrients) (St Leger *et al.*, 1989*a, c*), and as such *Metarhizium* can serve as a useful model system for understanding signal transduction and the control of growth and morphological differentiation in a fungus.

Signal reception for cell differentiation probably occurs soon after the second nuclear division since inhibition of synthesis of DNA or RNA prior to this event prevents appressorium formation (St Leger *et al.*, 1989*a*). The pathway of signal transduction of appressoria may involve a localized change in transmembrane potential produced by deformation of the plastic hyphal tip (St Leger *et al.*, 1990*a*). Germlings possess both a Ca^{2+} -dependent and a possible cAMP-dependent protein kinase activity suitable for signal mediation; the latter activity is associated with plasma membranes. Both types of kinase possess a large number of endogenous substrates (St Leger *et al.*, 1990*b*). Plasma membranes also contain a group of guanine-nucleotide-binding proteins suited to be coupling factors for transmembrane signal transduction (St Leger *et al.*, 1989*d*), a membrane-bound protein tyrosine kinase (St Leger *et al.*, 1989*c*), a phosphoprotein phosphatase (St Leger *et al.*, 1990*b*) and adenylate cyclase activity apparently involved in transmembrane signalling reactions, since mechanical or chemical treatments which stress the fungus or depolarize the plasma membrane cause rapid increases in intracellular levels of cAMP (St Leger *et al.*, 1990*b*). As in animal and plant systems, exocytosis requires the presence of Ca^{2+} (St Leger *et al.*, 1989*c*). These discoveries suggest a mechanism for a signal response by which an external stimulus is converted into intracellular second messengers, messenger-dependent regulatory phosphorylation activities, and finally to cell response. In the work presented here compounds that affect signalling and phosphorylation were used in order to test this conceptual framework with regard to polar growth and appressorium formation by *Metarhizium*.

Methods

Organism and growth. The fungal isolate (*Metarhizium anisopliae* ME1) and culture media were described by St Leger *et al.* (1987). Germination experiments and studies of differentiation in the presence of inhibitors were done in yeast extract medium (YEM, 0.0125% w/v) (St Leger *et al.*, 1989*d*).

Chemicals. ^{125}I -Labelled CaM and $^{45}\text{Ca}^{2+}$ in an aqueous solution of CaCl_2 [specific activity about 15 mCi mg^{-1} (555 MBq mg^{-1})] were from NEN. H-8 was from Seikagaku America Inc. (Sp)-cAMPS was from Boehringer Mannheim. Novozym 234 was from Novo Biolabs. Yeast extract was from Difco. Other chemicals were from Sigma.

Cyclic AMP content in differentiated germlings. Conidia suspended in YEM were germinated in 13.5 cm polystyrene dishes. After various periods, the cultures were harvested after pouring off the supernatant and washing the Petri dish bottom by stirring with distilled water (1 min). Ice-cold 5% (w/v) trichloroacetic acid (TCA) (5 ml) was added to a single dish, and the adherent germlings were scraped off. The TCA was then transferred sequentially to two other dishes and the mycelia were homogenized (1 min) using a motor-driven ($\sim 1100 \text{ r.p.m.}$) Teflon pestle and then extracted for a further 30 min at 0°C . In this way germlings from 15 dishes were pooled into five aliquots for assay. All subsequent steps were as described for conidia and mycelia (St Leger *et al.*, 1990*b*).

Localization of CaM. Conidia or germlings growing on polystyrene were fixed in 3.7% (v/v) formalin for 60 min, rinsed in sodium phosphate buffer (0.1 M, pH 7) and in 0.4 M-mannitol (pH 5.5), treated with 0.2% Novozym in 0.4 M-mannitol (30 min), rinsed in buffer and further permeabilized with 0.1% Triton X-100 (30 min). After washing with buffer the preparations were incubated for 10 min in an aqueous solution of the fluorescent CaM probe W-5 ($35 \mu\text{M}$) before visualization using a Zeiss microscope equipped with a 365 nm excitation filter and 395 nm dichroic mirror.

Uptake of $^{45}\text{Ca}^{2+}$. Aliquots (2 ml) of a conidial suspension (1×10^6 spores ml^{-1}) in YEM (0.0125%) were incubated at 27°C in 5.5 cm Petri dishes until about 50% of the germlings had undergone two mitotic divisions (after about 14 h). Cultures were supplemented with $^{45}\text{Ca}^{2+}$ [$3 \mu\text{Ci ml}^{-1}$ (111 kBq ml^{-1})] and following a further incubation period (10 h), the supernatant was poured off from each dish and the Petri dish bottom was washed by stirring with three changes of 10 mM-Tris/HCl buffer (pH 7.2) ($3 \times 3 \text{ ml}$, 2 min each). The Petri dishes were then extracted for 20 min with fresh Ca^{2+} -free Tris buffer or buffer supplemented either with CaCl_2 (5 mM) or EGTA (5 mM). Following extraction the Petri dishes were washed with two changes of Tris buffer and the adherent germlings under buffer were scraped off the dish bottom with a rubber policeman. The cells were filtered on a glassfibre (Whatman GF/C) disc and washed twice with Tris buffer at 0°C . The filters were dried and the radioactivity was determined with a scintillation counter. The amount of $^{45}\text{Ca}^{2+}$ binding to cells extracted with unsupplemented Tris buffer was taken to represent the total bound $^{45}\text{Ca}^{2+}$. The extent of decline in $^{45}\text{Ca}^{2+}$ binding after cells were extracted with Tris buffer supplemented with CaCl_2 or EGTA was taken as a measure of surface-bound $^{45}\text{Ca}^{2+}$ (after correction for background radioactivity on Petri dishes determined using dishes incubated with $^{45}\text{Ca}^{2+}$ -labelled YEM minus spores).

Ca^{2+} uptake during sporulation was studied using conidia obtained from a 12-d-old culture grown on Sabouraud dextrose agar supplemented with $^{45}\text{Ca}^{2+}$ ($3 \mu\text{Ci ml}^{-1}$). Samples (200 μl) of a conidial suspension ($3 \times 10^6 \text{ ml}^{-1}$) were spotted directly onto glassfibre discs. The conidia were washed ($5 \times 5 \text{ ml}$) with Tris buffer. The conidia were further washed in Tris buffer ($3 \times 5 \text{ ml}$) or Tris buffer supplemented with unlabelled CaCl_2 (5 mM) to remove surface-bound label. Residual cell-bound $^{45}\text{Ca}^{2+}$ was determined as described above.

Localization of Ca^{2+} within the cell. Conidia ($1 \times 10^6 \text{ ml}^{-1}$) were germinated for 32 h in YEM (0.05%, w/v) shake cultures (75 r.p.m.) in the presence of $^{45}\text{Ca}^{2+}$ ($3 \mu\text{Ci ml}^{-1}$). Mycelia were harvested by filtration (Whatman no. 1 paper), shaken for 30 min at 25°C in buffer (10 mM-Tris/HCl, 1 mM-EGTA), centrifuged (4000 g, 10 min) and the pellet washed in 1 M-sorbitol. Protoplasts were obtained by suspending mycelia in buffer (1.2 M-sorbitol, 10 mM-Tris/HCl, pH 7) supplemented with 0.8% Novozym 234 (Goettel *et al.*, 1990). Protoplasts were separated from cellular debris by filtration through cheesecloth and a $20 \mu\text{m}$ plastic screen. Following centrifugation (2000 g, 10 min) the protoplast pellet was washed three times with buffer. Protoplasts were suspended in 5 ml 330 mM-sucrose containing BSA (0.3%, w/w) (pH 7.1) and disrupted by sonication at 4°C . The homogenate was centrifuged (1000 g, 10 min) and a mitochondrial fraction was obtained from the supernatant by a further centrifugation (15000 g, 30 min). The 15000 g supernatants were recentrifuged (12000 g, 30 min) to remove remaining mitochondria, and the supernatants centrifuged a final time at 40000 g (40 min) to pellet plasma membranes (Bowman & Bowman, 1988). Mitochondrial and plasma-membrane pellets were suspended in 0.6 ml 10 mM-Tris/HCl buffer (pH 7.5) containing 1 mM-EGTA. The radioactivity in supernatants and pellets was determined using a scintillation counter.

Isolation of organelles for $^{45}\text{Ca}^{2+}$ influx studies. Mitochondria and vacuoles were isolated from mycelia from 32 h SDB cultures using the bead-beater procedure of Bowman & Bowman (1988). Mitochondria were separated from vacuoles by sucrose gradient centrifugation (Bowman & Bowman, 1988). Immediately thereafter the fractions were assayed for protein content (Bradford, 1976) and for Ca^{2+} transport activity.

Ca^{2+} uptake by isolated organelles. [Modified from Cornelius & Nakashima (1987) and Uchida *et al.* (1988).] $^{45}\text{Ca}^{2+}$ uptake by membrane fractions was measured at 22°C as follows. (1) For mitochondria the reaction mixture (100 μl) contained 5 mM-PIPES/Tris buffer (pH 7.8), 5 mM- Na_2ATP , 5 mM- MgSO_4 , 50 mM-KCl, 5 mM- K_2HPO_4 , 1 mM- Na_3VO_4 (to inhibit residual plasma-membrane ATPase) and 0.2 mM- CaCl_2 [containing 0.25 μCi (9.25 kBq) $^{45}\text{Ca}^{2+}$]. Uptake was initiated by adding 20 μg of mitochondria (determined by protein content). (2) For vacuoles the reaction mixture (100 μl) contained 5 mM-PIPES/Tris buffer (pH 7.4), 5 mM- Na_2ATP , 5 mM- MgCl_2 , 1 mM- Na_3VO_4 and 0.4 mM- CaCl_2 (containing 0.25 μCi $^{45}\text{Ca}^{2+}$). Uptake was initiated by adding 20 μg of vacuole membranes. Uptake was stopped after 10 min by collecting mitochondria or vacuoles onto 0.45 μm membrane filters and rapidly washing with two 5 ml aliquots of ice-cold buffer minus $^{45}\text{Ca}^{2+}$ and ATP. The filters were incubated in washing buffer for 30 min under vacuum prior to use. Radioactivity was determined in a liquid scintillation counter. Zero time controls were used as blanks.

Miscellaneous. Nuclei and cell walls were stained using DAPI and Uvitex, respectively (St Leger *et al.*, 1989a). The distribution of intracellular membranes was assessed with 3,3'-dihexylocarbocyanine iodide (Butt *et al.*, 1989). CTC staining of Ca^{2+} was done as described in Butt *et al.* (1989). CaM levels were determined with a CaM-specific radioimmunoassay (St Leger *et al.*, 1989c). ^{125}I -Labelled CaM binding was done as described by St Leger *et al.* (1990b). Proteins were processed for and subjected to SDS-PAGE as described by St Leger *et al.* (1989b). The $^{45}\text{Ca}^{2+}$ overlay method of Maruyama *et al.* (1984) was used to detect Ca^{2+} -binding proteins after separation by SDS-PAGE and transfer to nitrocellulose filters.

Antagonists were added to cultures from stock solutions in YEM. Ionophores were added from stock solutions in ethanol. The final concentration of ethanol was $\leq 0.04\%$ which had no effect on differentiation.

Results

Conidia germinating in 0.0125% YEM usually produced a single germ-tube slightly lateral to one of the poles. Non-differentiated *Metarhizium* germlings grow by apical extension of the hyphal tip until induced to undergo cell differentiation by contact with a suitable substrate (e.g. polystyrene) when germlings typically produced a terminal swelling (appressorium; Fig. 1a) the first in a series of structures normally involved in infection of a host.

Prior to germination, localized components of the cytoplasm must exist to orient the growth axis of the germling and initiate polarized outgrowth of the germ-tube. Potentially, localized cytoplasmic Ca^{2+} may orient the growth axis. However, most germinating conidia of *Metarhizium* were labelled uniformly with CTC, and less than 10% of spores displayed a non-uniform distribution of fluorescent intensity that would indicate a gradient of Ca^{2+} within the cell that might relate to polarization. Disruption of minute or otherwise undetectable Ca^{2+} gradients in conidia with high concentrations of the calcium ionophore Br-A23187 (50 μM) did not prevent polar outgrowth of germ-tubes, although germ-tubes were shorter and broader at the base which fluoresced brightly with Uvitex in the presence of ionophore (Fig. 1b, Table 1). At a lower concentration (10 μM) Br-A23187 increased the incidence of bipolar germination from 4% (in YEM controls) to 12% ($\chi^2 = 11.9$, $P < 0.01$). Over 80% of the conidia demonstrating bipolar germination produced germ-tubes at opposite poles of the spore (Fig. 1c), which suggested that bipolarity did not reflect a disruption of the growth axis. Usually, following nuclear division, only one of the germ-tubes received a daughter nucleus; the second smaller germ-tube remained anucleate and non-septate. As expected from previous results (St Leger *et al.*, 1989c) germination was normal in a Ca^{2+} -free medium (2 mM-EGTA) but was much reduced or abnormal with formation of a short bulbous germling when intracellular Ca^{2+} was depleted (Br-A23187 + 2 mM-EGTA) (Fig. 1d). In addition, while 50 μM -Br-A23187 (minus EGTA) reduced germination (i.e. the morphological expression of polarity) by only 10–15%, elongation of germ-tubes was reduced by 30% after 24 h (Table 1) and by over 70% after 30 h indicating that regardless of its role in fixing the growth axis, intracellular Ca^{2+} is important for the maintenance of rapid tip growth.

Membrane-associated Ca^{2+} in germlings was localized by CTC fluorescence. Intracellular Ca^{2+} may be demonstrated by weakly fluorescing punctate material observed in the cytoplasm of germlings. Unfortunately, because of the rapid bleaching of fluorescence (within seconds), it was not possible to identify the organelles responsible. A

Fig. 1. Fluorescence micrographs of *Metarhizium* germinated in YEM (0.0125%) on polystyrene for 24 h (unless stated otherwise). (a) Germling stained with Uvitex to show terminal appressorium. (b, c) Germlings grown with Br-A23187 and stained with Uvitex to show bulbous swelling at base of germ-tube (b; 50 μ M-Br-A23187) or bipolar germination (c; 10 μ M-Br-A23187). (d) Formation of short bulbous germlings (stained with Uvitex) in the presence of Br-A23187 (10 μ M) + EGTA (2 mM). (e) Localization of Ca^{2+} in a differentiated germling by CTC fluorescence. (f) Distribution of intracellular membranes assessed with 3,3-dihexylocarbocyanine iodide (note differential staining of appressoria). (g) Formation of short bulbous germlings (stained with Uvitex) in the presence of R24571 (3 μ M). (h, i) W-5-associated fluorescence in a permeabilized dry conidium (h) and a conidium presoaked in YEM (0.0125%) for 6 h (i); arrows indicate localization of CaM at conidial poles. (j) W-5-associated fluorescence of a germling grown for 40 h in 0.02% YEM; arrow indicates a subterminal appressorium. Bars, 10 μ m; C, conidium.

Table 1. Effects of Br-A23187 and second messengers on the growth of *Metarhizium*
Conidia were incubated for 24 h with inhibitor in YEM.

Br-A23187 (μM)	Addition	Percentage germination	Mean length ($\pm$ SE) of germ-tube (μm)	Percentage differentiation	Mean no. of growing points per germ-tube
0	0	>95	65 $\pm$ 7	68	1.04
0	EGTA (2 mM)	>95	61 $\pm$ 8	62	1.04
3	0	>95	60 $\pm$ 6	63	1.012
5	0	>95	58 $\pm$ 6	56	1.62
10	0	>95	52 $\pm$ 5	21	1.83
50	0	81	45 $\pm$ 6	0	1.10
5	EGTA (2 mM)	68	11 $\pm$ 2	0	1.0
0	CaCl ₂ (0.1 mM)	>95	68 $\pm$ 9	75	1.22
5	CaCl ₂ (0.1 mM)	>95	76 $\pm$ 7	61	1.21
0	CaCl ₂ (1 mM)	>95	76 $\pm$ 6	54	1.24
5	CaCl ₂ (1 mM)	>95	96 $\pm$ 8	0	1.13
0	CaCl ₂ (10 mM)	92	74 $\pm$ 7	52	1.12
5	CaCl ₂ (10 mM)	93	81 $\pm$ 5	0	1.05
0	CaCl ₂ (100 mM)	82	60 $\pm$ 10	5	1.06
5	CaCl ₂ (100 mM)	68	46 $\pm$ 8	0	1.04
5	MgCl ₂ (1 mM)	>95	75 $\pm$ 4	4	1.20
5	MgCl ₂ (10 mM)	>95	83 $\pm$ 14	0	1.04
3	cAMP (10 mM)	92	48 $\pm$ 3	0	1.86
3	cGMP (10 mM)	93	44 $\pm$ 8	0	1.92
3	(Sp)-cAMPS (1 mM)	90	49 $\pm$ 4	0	1.73
3	IBTX (10 mM)	94	47 $\pm$ 3	0	2.18
3	CaCl ₂ (1 mM) + cAMP (10 mM)	>95	82 $\pm$ 7	0	1.09
0	H-8 (1 mM)	>95	72 $\pm$ 6	0	1.04
5	H-8 (1 mM)	>95	70 $\pm$ 4	0	1.09

diffuse and more permanent cortical staining (Fig. 1e) may reflect Ca²⁺ associated with the mucus, appressorial wall or the plasmalemma. As CTC fluorescence is affected by membrane density (Caswell, 1979), the distribution of intracellular membranes was assessed with 3,3'-dihexylocarbocyanine iodide (Butt *et al.*, 1989). Preferential staining of appressoria (Fig. 1f) is indicative of the high density of endoplasmic reticulum and mitochondria these cells contain. However, fluorescence did not overlap with Ca²⁺-CTC fluorescence.

Recent studies indicate that the Ca²⁺-binding protein CaM is the regulator of Ca²⁺-dependent processes in *Metarhizium* conidia (St Leger *et al.*, 1989c, 1990b). Consistent with this, CaM antagonists such as R24571 inhibit germination or produce a swollen germ-tube resembling that formed by Ca²⁺ depletion (Fig. 1g). W-5, a specific fluorescent antagonist of CaM was used to localize CaM. W-5-associated fluorescence in permeabilized conidia was concentrated as spots at the very tips of the poles (Fig. 1h). Presoaking spores in YEM (0.0125%) for 6 h to swell them, an event that precedes normal germination, produced a more diffuse fluorescence, but the highest fluorescence intensity was still found in the region of the poles (Fig. 1i). Growing hyphae and appressoria showed a weaker, more uniform fluores-

cence (Fig. 1j). In treatments omitting permeabilization of conidia and germlings, hyphae were unstained except for a weak fluorescence at the periphery of cells. Thus, W-5 is not membrane permeable.

Germlings continued to produce high levels of appressoria in a Ca²⁺-free medium (2 mM EGTA) (Table 1) indicating that Ca²⁺ uptake is not necessary for signal mediation for appressorium formation. However, addition of Ca²⁺ (0.1 mM-CaCl₂, Fig. 2a) increased differentiation frequency at 24 h from 68% (YEM alone) to 76% ($\chi^2 = 4.76$, $P < 0.05$). At higher concentrations, Ca²⁺ lowered differentiation frequency (Table 1) and increased the proportion of appressoria with abnormal morphologies. Thus, CaCl₂ at 1 mM (Fig. 2b) and 10 mM (Fig. 2c) caused formation of narrow branched appressoria which were frequently subterminal or grew with a curved orientation and produced large amounts of mucus. CaCl₂ at 100 mM reduced polar growth and resulted in abnormally thin, frequently curved hyphae (Fig. 2d-e). Although mitotic division was not inhibited, large vacuoles (voids) in the cytoplasm were sometimes observed that were rarely seen in controls.

The effects of Ca²⁺ on differentiation were greatly potentiated by the presence of Br-A23187. At high concentrations ($\geq 10 \mu\text{M}$), the ionophore was an effective

Fig. 2. Fluorescence micrographs of *Metarhizium* germinated in YEM (0.0125%) on polystyrene for 24 h. (a–d) Germlings stained with Uvitex to show behaviour in the presence of (a) 0.1 mM- CaCl_2 , (b) 1 mM- CaCl_2 (note thin branched appressorium), (c) 10 mM- CaCl_2 (note curvilinear thin appressorium) or (d) 100 mM- CaCl_2 (note reduced growth, twin hyphae and inhibition of appressorium formation). (e). DAPI staining of germling grown in 100 mM CaCl_2 [note multiple nuclei (indicated by arrows) in a curved germ-tube]. (f) Uvitex staining of germling grown in 10 μM -Br-A23187 (note local ballooning of germ-tubes indicated by arrows). (g–i) Uvitex staining of germlings grown in 3 μM -Br-A23187 + 10 mM-cAMP [note bipolar germination (g, h), ballooning of germ-tube base (indicated by arrow) (g), and broad irregularly-shaped germ tube-with mucus (indicated by arrow) (i)]. Bars, 10 μm ; C, conidium.

inhibitor of appressorium formation, particularly in the presence of EGTA (Table 1). By contrast, Br-A23187 stimulated side branching (Table 1). Also, in the presence of ionophore about 40% of germ-tubes had

irregularly shaped cell walls and bulbous swellings along the hyphae apparently caused by periodic ballooning of the hyphal tips (Fig. 2f).

These effects may result from loss of intracellular Ca^{2+}

Fig. 3. Time course of changes in endogenous cAMP in germinating conidia producing non-differentiated germ-tubes (○) and in germ-lings forming appressoria (●). Each experiment was repeated three times. Results with differentiating cells are from the two experiments which produced the most disparate results (●—●, ●---●). Each point is the mean of five replicates $\pm$ SE. The time course for germination (Δ) and production of appressoria ($\blacktriangle$) is shown.

as application of ionophore simultaneously with exogenous Ca^{2+} (0.5 mM- CaCl_2) substantially increased polar growth (Table 1) resulting in long thin hyphae with smooth walls. The growing tips stained brightly with Uvitex indicative of plastic primary wall material, but differentiation of appressoria was entirely inhibited by an increased cytoplasmic Ca^{2+} concentration. Polar growth was stimulated to a lesser extent by Mg^{2+} plus A23187 or Br-A23187 (Table 1). However, addition of other divalent cations (Ba^{2+} , Zn^{2+} and Mn^{2+}) with A23187 had no visible effects on germ-tube elongation or else they were toxic (data not presented). Above 1 mM, Ca^{2+} was inhibitory to growth in the presence of ionophore (Table 1).

Levels of cAMP remained almost constant in non-differentiated germ-lings (6–24 h, post-inoculation) at about 0.4 ± 0.06 pmol (mg dry wt) $^{-1}$, respectively. cAMP levels in differentiating germ-lings fluctuated but at a level higher than in non-differentiated cells (Fig. 3).

Application of cyclic nucleotides (cAMP, cGMP), (Sp)-cAMPS [a phosphodiesterase-resistant, membrane-permeable activator of cAMP-dependent protein kinases (Rothermel *et al.*, 1984)] and the cyclic nucleotide phosphodiesterase inhibitor IBTX were ineffective at altering growth and morphology at any concentration used (0.1–10 mM). However, when applied simultaneously with Br-A23187, they potentiated the effects of the ionophore (Table 1). Thus, although Br-A23187 at 3 μM has little apparent effect on morphology by itself, when combined with either cAMP, cGMP or IBTX (all at 10 mM) or (Sp)-cAMPS (1 mM) it resulted in frequent (>30%) bipolar germination. In contrast to treatments with ionophore alone (Fig. 1c), germ-tubes formed from any part of the spore and were usually septate (Fig. 2g–i). Increased synthesis of mucus induced by Br-A23187 plus cAMP resulted in a high background with DAPI

staining but careful focusing revealed that the germ-tubes were multinucleate even when a nucleus remained in the conidium. The most likely explanation for this is that cyclic nucleotide stimulates the conidial nucleus to undergo two mitotic divisions; in controls (YEM only) the single conidial nucleus typically divides once and only the daughter nucleus which enters the growing germ-tube divides again (St Leger *et al.*, 1989a). Germ-lings that formed in the presence of Br-A23187 plus cAMP were short and often uniformly broad, so that distinguishable appressoria could not be observed. Occasionally, a balloon-like swelling formed from the conidia before the germ-ling was successful in establishing polarized tip growth (Fig. 2g). Production of side-branches on the short germ-tubes was also indicative of multiple attempts to form apices. The walls of the germ-lings fluoresced brightly with Uvitex, particularly in those regions which were irregularly shaped or swollen.

Application of exogenous Ca^{2+} counteracted the effects of ionophore plus cAMP as effectively as it overcame growth inhibition produced by treatment with the ionophore alone (Table 1). Thus, perturbation of the intracellular Ca^{2+} gradient is necessary before the effects of cAMP are expressed. Conversely, formation of side-branches and appressoria by germ-lings is repressed by H-8 [the H-series inhibitor with the highest affinity for cyclic nucleotide dependent kinases (Hidaka & Tanaka, 1987)] in the presence or absence of ionophore.

Ca^{2+} - and CaM-binding proteins

CaM levels (radioimmunoassay) in non-differentiated and differentiated germ-lings were not significantly different at 3.2 ± 0.68 and 3.7 ± 0.45 μg rate equivalent CaM (mg total protein) $^{-1}$, respectively. Several prominent target proteins were found in differentiated germ-lings. However, there were no reproducible differences with the ^{125}I -labelled CaM-binding proteins detected in ungerminated germ-lings (data not presented). The autoradiograph in Fig. 4 shows the binding of $^{45}\text{Ca}^{2+}$ to proteins from differentiated cells. Electrophoresis was done in the presence of CaCl_2 (5 mM) or EGTA (5 mM) since some Ca^{2+} -binding proteins (e.g. CaM) have increased migration rates in the presence of Ca^{2+} . Two proteins migrated faster in the presence of Ca^{2+} ; other Ca^{2+} -binding proteins co-migrated with proteins incubated with EGTA.

Ca^{2+} uptake and distribution in *Metarhizium*

$^{45}\text{Ca}^{2+}$ accumulated in conidia when mycelia sporulated on Sabouraud dextrose agar containing the

Fig. 4. Ca^{2+} -binding proteins in differentiated germlings of *Metarhizium*. Proteins were separated by SDS-PAGE in the presence of 5 mM-EGTA (lane 1) or 5 mM- CaCl_2 (lane 2), transferred to a 0.2 μm nitrocellulose membrane and exposed to $^{45}\text{Ca}^{2+}$ (1 $\mu\text{Ci ml}^{-1}$) in the presence of 60 mM-KCl and 5 mM-Mg Cl_2 . Arrowheads indicate proteins with increased mobility in the presence of Ca^{2+} .

isotope. Washing conidia in Tris buffer plus unlabelled CaCl_2 (5 mM) lowered cell-bound $^{45}\text{Ca}^{2+}$ to 84000 ± 9000 c.p.m. per 6×10^5 conidia compared with 102000 ± 6000 c.p.m. per 6×10^5 conidia with Tris buffer alone, indicating that a component (about 18%) of the $^{45}\text{Ca}^{2+}$ was surface-bound. $^{45}\text{Ca}^{2+}$ uptake by germlings was studied during the period (14–24 h post-inoculation) of appressorium formation (Table 2). During differentiation, appressoria took up high levels of $^{45}\text{Ca}^{2+}$. A proportion (about 40%) of the $^{45}\text{Ca}^{2+}$ was removed by treatment with EGTA and was exchangeable with an excess of unlabelled Ca^{2+} , consistent with an accumulation of Ca^{2+} by the outer surface of the plasma membrane and the cell wall.

The cellular distribution of $^{45}\text{Ca}^{2+}$ was studied by cell fractionation and subsequent analysis of $^{45}\text{Ca}^{2+}$ in the cell fractions (Table 3). A decrease in the radioactivity of the particulate fraction occurred after cell wall digestion indicating the presence of Ca^{2+} in the cell walls. This decrease may have been underestimated due to redistribution of wall-bound Ca^{2+} following digestion. The association of $^{45}\text{Ca}^{2+}$ with the sedimented mitochondria/vacuole and plasma membrane fractions and lack

Table 2. $^{45}\text{Ca}^{2+}$ remaining bound to differentiated germlings after extraction of cell-wall-bound $^{45}\text{Ca}^{2+}$ with unlabelled CaCl_2 or EGTA

The values represent the amount of $^{45}\text{Ca}^{2+}$ remaining after extraction of germlings differentiating on a polystyrene dish.

Extraction medium	Growth medium	$10^{-3} \times \text{bound } ^{45}\text{Ca}^{2+}$ (c.p.m. per dish $\pm$ SE; $n = 5$)	
		YEM (0.0125%)	YEM (0.0125%) + CaCl_2 (1 mM)
10 mM-Tris/HCl (pH 7.2)		345.5 ± 62	13.0 ± 4
10 mM-Tris/HCl (pH 7.2) + 5 mM- CaCl_2		193.2 ± 34	6.9 ± 2
10 mM-Tris/HCl (pH 7.2) + 5 mM-EGTA		183.7 ± 17	7.4 ± 2

Table 3. $^{45}\text{Ca}^{2+}$ in fractions of *Metarhizium mycelia*

Fraction	$10^{-3} \times ^{45}\text{Ca}^{2+}$ content (c.p.m. ml^{-1})
Intact mycelia (100 mg wet wt)	154
Protoplasts (derived from 100 mg wet wt of mycelia)	82
Low speed supernatant (15000 g) of protoplast lysate	29
Mitochondrial pellet	65
High speed supernatant (40000 g) of protoplast lysate	2.6
Plasma membrane pellet	24

of radioactivity in the supernatants indicates that $^{45}\text{Ca}^{2+}$ was tightly bound to these structures.

Both mitochondria and vacuoles sequestered $^{45}\text{Ca}^{2+}$ in the presence of permeant precipitating anions in a process stimulated by MgATP and inhibited by DCCD, an inhibitor of H^+ -translocating ATPases (Table 4). Under the conditions of our assay, vacuoles took up $^{45}\text{Ca}^{2+}$ at a 25-fold greater rate than mitochondria.

The role of Ca^{2+} channels in differentiation

The role of Ca^{2+} channels and the endoplasmic reticulum were studied using well-characterized inhibitors. Plasma membrane Ca^{2+} channel blockers (nifedipine or verapamil) at 50 μM or specific antagonists of Ca^{2+} transport across endoplasmic reticulum [ryanodine (50 μM), fluorescein isothiocyanate (100 μM) and TMB-8 (100 μM)] did not affect differentiation frequency. In fungi and other organisms, Li^+ ions (≤ 5 mM) specifically disrupt the phosphatidylinositol cycle which mediates the release of Ca^{2+} from the endoplasmic reticulum and

Table 4. $^{45}\text{Ca}^{2+}$ uptake by mitochondria and vacuoles

Inhibitor	$10^{-3} \times ^{45}\text{Ca}^{2+}$ uptake [c.p.m. ($\mu\text{g protein})^{-1} \pm \text{SE}$]*	
	Mitochondria	Vacuoles
None	3.2 ± 0.4 (100)	78.4 ± 14.0 (100)
DCCD (0.01 mM)	0.45 ± 0.1 (14)	17.53 ± 5.7 (22)
Na_3VO_4 (0.1 mM)	2.94 ± 0.4 (92)	69.26 ± 6.3 (90)
($-\text{Mg}^{2+}$)†	1.34 ± 0.3 (42)	28.20 ± 8.2 (36)
($-\text{ATP}$)†	0.90 ± 0.2 (28)	25.54 ± 5.4 (33)

* Percentage values are given in parentheses.

† MgCl or ATP were omitted from reaction mixtures.

activates protein kinase C (Hanson, 1989; Van Lookeren Campagne *et al.*, 1988). High levels of LiCl (> 20 mM) were required to inhibit growth and differentiation of *Metarhizium*. At 10 mM, differentiation frequency was reduced by only 9% from controls.

Discussion

The Ca^{2+} -CTC membrane associated fluorescence in tip-growing systems and the stimulation of polar growth by Ca^{2+} has led to the conclusion that a tip-to-base Ca^{2+} -gradient is involved specifically in apical extension (Jackson & Heath, 1989; Picton & Steer, 1983). The effects of Br-A23187 demonstrate that tip growth and differentiation of appressoria in *Metarhizium* are also very sensitive to changes in the distribution of Ca^{2+} and perhaps Mg^{2+} . Most studies to date have not tested Mg^{2+} even though the ionophore used (A23187) is less specific for Ca^{2+} , as compared with Mg^{2+} , than the brominated derivative (Br-A23187) used in this study (Deber *et al.*, 1985). In *Metarhizium* the effects of Mg^{2+} may complement those of Ca^{2+} as its adenylate cyclase and the Ca^{2+} -dependent kinases have a requirement for Mg^{2+} (St Leger *et al.*, 1990b) as does Ca^{2+} uptake by vacuoles.

Inhibition of germination by Ca^{2+} deprivation and the multiple emergence of broad and branched germ-tubes from conidia in the presence of Br-A23187 indicates that while Ca^{2+} plays a fundamental role in tip growth, perhaps establishing the dominance of apical growth (Schmid & Harold, 1988), it may not be involved directly in initiating polarity and formation of an apex. The effects of Ca^{2+} may be mediated by other components of the cell. The relative inability of Ca^{2+} in the absence of Br-A23187 to affect cell growth argues against an important direct effect of Ca^{2+} on the *Metarhizium* cell wall. High concentrations of Ca^{2+} , however, prevented lateral expansion of the apical tip, which, by analogy with plant systems (Demarty *et al.*, 1984) may result from

a stiffening of the cell wall produced by the accumulation of Ca^{2+} . An increase in the intracellular Ca^{2+} concentration will stimulate the action of the Ca^{2+} - and Ca^{2+} /CaM-dependent kinases which predominate in the cytoplasm of *Metarhizium* and consequently produce a biochemical cascade (St Leger *et al.*, 1990b). Consistent with this, the localization of CaM at the poles of the conidium implies an important CaM-mediated role for Ca^{2+} in the manifestation of polarity as suggested in tip-growing plant cells (Hauber *et al.*, 1984). CaM-dependent phosphorylation events are crucial for the nutrient uptake and protein synthesis required for germination of *Metarhizium* conidia (St Leger *et al.*, 1989c). In later stages, CaM might then help establish polarity by interacting with the cytoskeleton to regulate a variety of intracellular processes such as cytoplasmic streaming and vesicle transport. Inhibition by CaM antagonists of tip-growth and the production of bulbous swellings from conidia is consistent with such a role. We found no pharmacological evidence for phosphoinositide or protein kinase C signalling or a role for the endoplasmic reticulum as a Ca^{2+} store during germination and differentiation of *Metarhizium*.

There is an apparent contradiction in the roles Ca^{2+} may play during appressorium formation. The activation of a secretory process in animal and plant cells is generally linked to an increase in the cytoplasmic Ca^{2+} concentration. In *Metarhizium* too, secretion is a Ca^{2+} -dependent process and germ-tube tips and appressoria are very active secretors of mucus and enzymes (Goettel *et al.*, 1989; St Leger *et al.*, 1987, 1989b). Also, appressorium formation was more sensitive to disruption of intracellular Ca^{2+} gradients than was polar growth. However, by analogy with other systems (Jackson & Heath, 1989; Picton & Steer, 1982) an increase in the cytoplasmic Ca^{2+} concentration would prevent the relaxation of the actin cytoskeleton necessary for lateral expansion of the apical tip. An obligatory role for actin in tip growth of *Metarhizium* and the transition to non-polarized growth was shown by depletion of actin filaments with cytochalasin A (St Leger *et al.*, 1990a). Also, consistent with this, increasing the Ca^{2+} concentration by ionophoresis stimulated polar growth at the expense of appressorial formation while endogenous reserves of Ca^{2+} , originating from the conidium, are sufficient for differentiation. The ability of the mitochondria and vacuoles to sequester Ca^{2+} suggests that these organelles, which are particularly large and abundant in appressoria (Zacharuk, 1970; Goettel *et al.*, 1989) may function to maintain localized cytoplasmic Ca^{2+} at the levels required for alterations in the cytoskeleton and other growth processes, as reported in plant systems (Picton & Steer, 1985). Consistent with this, mutants of *Neurospora crassa* defective in the

transport of Ca^{2+} into a vacuolar fraction are subject to inhibition by extracellular Ca^{2+} (Cornelius & Nakashima, 1987). The sensitivity of Ca^{2+} uptake by *Metarhizium* vacuoles to ATP, Mg^{2+} and DCCD resembles reports for yeast vacuoles in which the driving force for uptake is a pH gradient produced by a H^{+} -translocating ATPase that uses MgATP as a substrate (Uchida *et al.*, 1988). The presence of CaM- and other Ca^{2+} -binding proteins in *Metarhizium* appressoria provides an additional energy-independent component of Ca^{2+} buffering in the cytoplasm. Enhanced exocytosis with low cytoplasmic levels of Ca^{2+} may be possible if the plasma membrane and bordering cell wall are a localized reservoir of Ca^{2+} (as suggested by the distribution of ^{45}Ca and also by CTC staining) and if the effects of the small localized changes in Ca^{2+} concentration that effect apical dominance are potentiated by another second messenger such as cAMP. This possibility was confirmed by the effects of cAMP when added to otherwise ineffective concentrations of Br-A23187. In combination cAMP (or IBTX) and Br-A23187 generate an extra mitotic division in the conidia, reduce polar growth and stimulate secretion of mucus. cGMP had similar effects except that production of mucus was less. In some aspects, the morphological effects of cAMP in combination with Br-A23187 mimic the formation of appressoria, notably the swelling of the cell with concomitant reduction in extension growth, enhanced Uvitex staining of the cell wall and enhanced mucus production. The results with Uvitex suggest that changes in cAMP concentration may have an effect on the organization of wall formation, which is fundamental to differentiation of *Metarhizium* (St Leger *et al.*, 1990a). In contrast to our observations with *Metarhizium*, cAMP represses formation of side-branching in *Neurospora crassa* (Reissig & Kinney, 1983). It is, however, possible that fluctuations in the relative concentrations of Ca^{2+} and cAMP are the controlling influence on morphology as high concentrations of ionophore or ionophore plus Ca^{2+} did not allow branching in the presence of cAMP.

The fluctuating rise in cAMP concentrations recorded during differentiation is circumstantial evidence that cAMP has a role in appressorium formation. These prolonged shifts, lasting hours, are unlike the transient responses previously recorded in *Metarhizium* (St Leger *et al.*, 1990b). They resemble, however, long-term changes which occur in several dimorphic fungi (e.g. *Histoplasma capsulatum*; Maresca & Kobayashi, 1989). Better evidence for a role for cAMP in differentiation was provided by application of H-8, previously shown to inhibit cAMP-sensitive phosphorylation at the plasma membrane and phosphorylation of a 27 kDa cytoplasmic protein (St Leger *et al.*, 1989c, 1990a). Cells whose differentiation was blocked with H-8 produced little

mucus and adhered poorly to the substrate. This suggests that cAMP-stimulated mucus secretion is responsible for adhesion. It is also possible that adhesion is a prerequisite for differentiation or that the mucus contains components required for signal reception. Together with our previous evidence that treatments which stress the fungi or depolarize the plasma membrane cause rapid increases in intracellular cAMP levels (St Leger *et al.*, 1990b) these results suggest that cAMP may play a role in the mediation of externally determined differentiation processes.

This work was supported in part by a grant (89-37263-4463) from the USDA Competitive Research Grants Office.

References

- BOWMAN, E. J. & BOWMAN, B. J. (1988). Purification of vacuolar membrane, mitochondria and plasma membranes from *Neurospora crassa* and modes of discriminating among the different H^{+} -ATPases. *Methods in Enzymology* **157**, 562–573.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- BUTT, T. M., HOCH, H. C., STAPLES, R. C. & ST LEGER, R. J. (1989). Use of fluorochromes in the study of fungal cytology and differentiation. *Experimental Mycology* **13**, 303–320.
- CASWELL, A. H. (1979). Methods of measuring intracellular calcium. *International Review of Cytology* **56**, 145–181.
- CORNELIUS, G. & NAKASHIMA, H. (1987). Vacuoles play a decisive role in calcium homeostasis in *Neurospora crassa*. *Journal of General Microbiology* **133**, 2341–2347.
- DEBER, C. M., TOM-KUN, J., MACK, E. & GRINSTEIN, S. (1985). Bromo-A23187: a nonfluorescent calcium ionophore for use with fluorescent probes. *Analytical Biochemistry* **146**, 349–352.
- DEMARTY, M., MORVAN, C. & THELLIER, M. (1984). Calcium and the cell wall. *Plant, Cell and Environment* **7**, 441–448.
- GERISCH, G. (1987). Cyclic AMP and other signals controlling cell development and differentiation in *Dictyostelium*. *Annual Review of Biochemistry* **56**, 853–879.
- GOETTEL, M. S., ST LEGER, R. J., RIZZO, N. W., STAPLES, R. C. & ROBERTS, D. W. (1989). Ultrastructural localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta*) cuticle. *Journal of General Microbiology* **134**, 2233–2239.
- GOETTEL, M. S., ST LEGER, R. J., BHAI, S., JUNG, M. K., OAKLEY, B. R., ROBERTS, D. W. & STAPLES, R. C. (1990). Transformation of the entomopathogenic fungus *Metarhizium anisopliae* using the *Aspergillus nidulans* *benA3* gene. *Current Genetics* **17**, 129–132.
- HANSON, B. A. (1989). The effects of lithium on the growth, morphology and biochemistry of *Neurospora crassa*. *Program of the XV Fungal Genetics Conference*, University of California, Los Angeles.
- HAUBER, I., HERTH, W. & REISS, H. D. (1984). Calmodulin in tip-growing cells, visualized by fluorescing calmodulin-binding phenothiazines. *Planta* **162**, 33–39.
- HIDAKA, H. & TANAKA, T. (1987). Transmembrane Ca^{2+} signalling and a new class of inhibitors. *Methods in Enzymology* **139**, 570–582.
- JACKSON, S. L. & HEATH, I. B. (1989). Effects of exogenous calcium ions on tip growth, intracellular Ca^{2+} concentration and actin arrays in hyphae of the fungus *Saprolegnia ferax*. *Experimental Mycology* **13**, 1–12.

- MARESCA, B. & KOBAYASHI, G. S. (1989). Dimorphism in *Histoplasma capsulatum*: a model for the study of cell differentiation in pathogenic fungi. *Microbiological Reviews* **53**, 186–209.
- MARUYAMA, K., MIKAWA, T. & EBASHI, S. (1984). Detection of calcium binding proteins by $^{45}\text{Ca}^{2+}$ autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. *Journal of Biochemistry* **95**, 511–519.
- McKERRACHER, L. J. & HEATH, I. B. (1987). Cytoplasmic migration and intracellular organelle movements during tip growth of fungal hyphae. *Experimental Mycology* **11**, 79–100.
- PICTON, J. M. & STEER, M. W. (1982). A model for the mechanism of tip extension in pollen tubes. *Journal of Theoretical Biology* **98**, 15–20.
- PICTON, J. M. & STEER, M. W. (1983). Evidence for the role of Ca^{2+} ions in tip extension in pollen tubes. *Protoplasma* **115**, 11–17.
- PICTON, J. M. & STEER, M. W. (1985). The effects of ruthenium red, lanthanum, fluorescein isothiocyanate and trifluoperazine on vesicle transport, vesicle fusion and tip extension in pollen tubes. *Planta* **163**, 20–26.
- REISSIG, J. L. & KINNEY, S. G. (1983). Calcium as a branching signal in *Neurospora crassa*. *Journal of Bacteriology* **154**, 1397–1402.
- ROTHERMEL, J. D., PERILLO, N. L., MARKS, J. S. & PARKER BOTELHO, L. M. (1984). Effects of the specific cAMP antagonist (Rp)-Adenosine cyclic 3',5'-phosphorothioate, on the cAMP-dependent protein kinase-induced activity of hepatic glycogen phosphorylase and glycogen synthase. *Journal of Biological Chemistry* **259**, 15294–15300.
- SCHMID, J. & HAROLD, F. M. (1988). Dual roles for calcium ions in apical growth of *Neurospora crassa*. *Journal of General Microbiology* **134**, 2623–2631.
- ST LEGER, R. J., COOPER, R. M. & CHARNLEY, A. K. (1987). Production of cuticle-degrading enzymes by the entomopathogen *Metarhizium anisopliae* during infection of cuticles from *Calliphora vomitoria* and *Manduca sexta*. *Journal of General Microbiology* **133**, 1371–1382.
- ST LEGER, R. J., BUTT, T. M., ROBERTS, D. W. & STAPLES, R. C. (1989a). Production *in vitro* of appressoria by the entomopathogenic fungus *Metarhizium anisopliae*. *Experimental Mycology* **13**, 274–288.
- ST LEGER, R. J., BUTT, T. M., STAPLES, R. C. & ROBERTS, D. W. (1989b). Synthesis of proteins including a cuticle degrading protease during differentiation of the entomopathogenic fungus *Metarhizium anisopliae*. *Experimental Mycology* **13**, 253–262.
- ST LEGER, R. J., ROBERTS, D. W. & STAPLES, R. C. (1989c). Calcium and calmodulin-mediated protein synthesis and protein phosphorylation during germination, growth and protein production by *Metarhizium anisopliae*. *Journal of General Microbiology* **135**, 2141–2154.
- ST LEGER, R. J., ROBERTS, D. W. & STAPLES, R. C. (1989d). Novel GTP-binding proteins in plasma membranes of the fungus *Metarhizium anisopliae*. *Biochemical and Biophysical Research Communications* **164**, 362–366.
- ST LEGER, R. J., ROBERTS, D. W. & STAPLES, R. C. (1990a). A model to explain differentiation of appressoria by germings of *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* (in the Press).
- ST LEGER, R. J., LACCETTI, L. B., STAPLES, R. C. & ROBERTS, D. W. (1990b). Protein kinases in the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of General Microbiology* **136**, 1401–1411.
- TAKEUCHI, Y., SCHMID, J., CALDWELL, J. H. & HAROLD, T. M. (1988). Transcellular ion currents and extension of *Neurospora crassa* hyphae. *Journal of Membrane Biology* **101**, 33–41.
- UCHIDA, E., OHSUMI, Y. & ANRAKA, Y. (1988). Purification of yeast vacuolar membrane H^{+} -ATPase and enzymological discrimination of three ATP-driven proton pumps in *Saccharomyces cerevisiae*. *Methods in Enzymology* **157**, 544–562.
- VAN LOOKEREN CAMPAGNE, M. M., GRNEUX, C., VAN EIJK, R. & VAN HANSTERT, P. J. M. (1988). Two dephosphorylation pathways of inositol 1,4,5-triphosphate in *Dictyostelium discoideum* homogenates. *Biochemical Journal* **254**, 343–350.
- WESSELS, J. G. H. (1986). Cell wall synthesis in apical hyphal growth. *International Review of Cytology* **104**, 37–79.
- ZACHARUK, R. Y. (1970). Fine structure of the fungus *Metarhizium anisopliae* infecting three species of larval elateridae (coleoptera). II. Conidial germ tubes and appressoria. *Journal of Invertebrate Pathology* **15**, 81–91.