Calcium- and Calmodulin-mediated Protein Synthesis and Protein Phosphorylation during Germination, Growth and Protease Production by *Metarhizium anisopliae*

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Protein synthesis and phosphorylation were studied in *Metarhizium anisopliae*. Calmodulin (CaM) and CaM-target proteins were found in conidia and germings of *M. anisopliae*. Conidial uptake of [³⁵S]methionine and [³²P]orthophosphate and their incorporation into protein was massively reduced by known antagonists of CaM, depletion of intracellular Ca²⁺ by ionophoresis or antagonism of Ca²⁺ with La³⁺, agents which prevented nuclear division and germination. Inhibitors of C-kinase (H-7) and cyclic-nucleotide-dependent kinase (H-8) selectively repressed phosphorylation of a 27 kDa protein but did not affect the profile of protein synthesis nor change germination frequency and mode of growth. By contrast, inhibitor and ionophoresis studies on mycelia showed that extracellular secretion of proteins but not protein synthesis was Ca²⁺-dependent. Protein phosphorylation in mycelia was also Ca²⁺-dependent/CaM-independent. CaM antagonists stimulated phosphorylation of 17 and 33 kDa polypeptides. Most proteins in mycelia were phosphorylated at serine and threonine residues. However, immunoblotting with anti-phosphotyrosine serum revealed prominent bands at 34.5 and 38 kDa. The 38 kDa protein was detectable on isolated plasma membranes. Our results suggest that *M. anisopliae* possesses stimulus transduction pathways similar to those known in plant and animal systems.

INTRODUCTION

Protein phosphorylation is recognized as the primary mechanism for the transduction of extracellular stimulants into intracellular events (Cohen, 1982; Nestler *et al.*, 1984). In animal systems, receptors for a variety of growth factors are themselves protein-tyrosine kinases (Sibley *et al.*, 1987). Furthermore, in higher eukaryotes the biochemical events produced by cyclic AMP (cAMP) are accomplished by activation of cAMP-dependent protein kinase (Krebs & Beavo, 1979). Ca²⁺ also regulates a diverse array of cellular functions by its ability to activate several protein kinases (Cohen, 1985). Many of the effects of Ca²⁺ are mediated by the Ca²⁺-binding protein calmodulin (CaM), which, in its Ca²⁺-activated form, is able to activate or inactivate a number of enzymes including protein kinases and at least one phosphoprotein phosphatase (Nairn *et al.*, 1985).

Information on the role(s) of Ca²⁺ and CaM in growth and development of fungi is sparse and indirect (Schmid & Harold, 1988; Giannini *et al.*, 1988). The presence in *Neurospora crassa* of CaM (Ortega Perez *et al.*, 1981), Ca²⁺ and CaM-dependent protein kinases (Ortega Perez *et al.*,

Abbreviations: Anti-PTYR antibodies, anti-phosphotyrosine antibodies; Br-A23187, 4-bromo-A23187; CaM, calmodulin; DAPI, 4,6-diamidino-2-phenylindole; RIA, radioimmunoassay; SDB(A), Sabouraud dextrose broth (agar); TPA, 12-*O*-tetradecanoylphorbol-13-acetate; YEM, yeast extract medium.

1983; Van Tuinen *et al.*, 1984) and a Ca^{2+} - and phospholipid-dependent protein kinase (protein kinase C) (Favre & Turian, 1987) indicates that Ca^{2+} has a regulatory role in this fungus. CaM appears to be ubiquitous in fungi (Muthukumar *et al.*, 1987) and is crucial in yeasts where it may be involved in the normal function of microtubules (Eilam & Chernichovsky, 1988). No distinctive function has yet been assigned to CaM in filamentous fungi.

If fungal cells use Ca^{2+} as a second messenger in a manner similar to animal cells (Carafoli, 1987) and plant cells (Hepler & Wayne, 1985), then protein-phosphorylation events should be sensitive to effectors of intracellular levels of Ca^{2+} and Ca^{2+} -binding proteins such as CaM. In this paper we report that calcium and CaM are required for protein synthesis and phosphorylation during germination of conidia of the entomopathogenic fungus *Metarhizium anisopliae*. We also investigated phosphorylation events during vegetative growth in conditions favourable to secretion of high levels of alkaline protease (Pr1), a pathogenicity determinant in *M. anisopliae* (St Leger *et al.*, 1988). Pr1 production was used as a model system to investigate the effects of Ca^{2+} on protein synthesis and processing, our eventual aim being to study the regulation of transcription and translation in the fungal cell by manipulating cellular levels of secondary messengers such as Ca^{2+} and cAMP.

METHODS

Organism and growth. The fungal isolate (*Metarhizium anisopliae* ME1) and culture media were described by St Leger *et al.* (1987). Germination experiments were done in yeast extract media (YEM; 0.0125%) (St Leger *et al.*, 1989a).

Chemicals. Affinity purified anti-phosphotyrosine antibodies (anti-PTYR antibodies) were a generous gift from Dr Jean Y. J. Wang (University of California, La Jolla, CA, USA). Other reagents were procured as follows: biotinylated CaM (Biomedical Technologies); ^{125}I -Protein A and [^{35}S]methionine (ICN); carrier-free [^{32}P]phosphoric acid and the CaM radioimmunoassay kit (Amersham); nitrocellulose (BA85, Schleicher and Schuell); protein kinase inhibitor H-8 (Seikagaku America); Sabouraud dextrose broth (SDB; Oxoid). Other chemicals were from Sigma.

Immunological detection of phosphotyrosine-containing proteins. Plasma membranes (about 30 μg protein) were vigorously shaken in 10 mM-Tris/HCl (pH 7.5) with or without Triton X-100 (0.1%, v/v) (final volume 50 μl). After 15 min an equal volume of electrophoresis sample buffer (Laemmli, 1970) was added and the preparation was heated for 5 min in a boiling water-bath. The samples were centrifuged in an Eppendorf centrifuge and the supernatants were analysed by SDS-PAGE (13%, w/v, acrylamide). Proteins were electrophoretically transferred from the gel to nitrocellulose and probed with anti-PTYR antibodies, followed by ^{125}I -protein A. Except for the specificity of the antibodies, the procedure was similar to that described previously (St Leger *et al.*, 1989b). Bovine serum albumin (3%, w/v) was used as blocking agent (Burnette, 1981). To differentiate between phosphorylation on serine and threonine residues versus tyrosine residues, gels were incubated in 1 M-NaOH at 55 °C (Cooper & Hunter, 1981).

CaM levels in conidia and mycelia. CaM levels were determined with a CaM-specific radioimmunoassay (RIA) kit. The specificity of this assay for fungal CaMs has been demonstrated previously (Muthukumar *et al.*, 1987).

Washed conidia (500 mg) harvested from 10-d-old SDA cultures (St Leger *et al.*, 1989a) and acid-washed sand (500 mg) (<0.3 mm diameter) were vortexed under liquid nitrogen in a 50 ml screwtop centrifuge tube for five periods of 5 min each. The fractured conidia and sand were then suspended in the Amersham CaM-extraction buffer supplemented with 3 mM-EDTA, 3 mM-EGTA and 0.5 mM-PMSF. All subsequent steps were as described in the RIA kit.

Mycelium (1 g wet wt) from 32 h SDB cultures was washed three times with 0.2 M-potassium phosphate buffer (pH 6.5), resuspended in the amended Amersham extraction buffer and disrupted for 3 min at 4000 r.p.m. in a Braun MSK homogenizer with 0.3 mm diameter glass beads. All subsequent steps were as described in the RIA kit.

The concentration of *Metarhizium* CaM was determined from a standard curve constructed using the rat CaM provided in the RIA kit. Results are given as μg rat equivalent CaM ($\text{mg protein}^{-1} \pm \text{SE}$ ($n = 5$)). Each result is the mean of five determinations made from different batches of conidia or mycelia from different flasks.

Labelling of CaM-binding proteins in protein blots (after Billingsley *et al.*, 1985). Proteins were isolated and separated by SDS-PAGE (13%, w/v, acrylamide) (St Leger *et al.*, 1989a), transferred to nitrocellulose and probed for 1 h with biotinylated CaM (10 $\mu\text{g ml}^{-1}$) in buffer (50 mM-Tris/HCl, pH 7.4, 300 mM-NaCl, 1 mM- CaCl_2 , 5%, w/v, nonfat dry milk). Specific CaM-binding proteins were visualized with avidin-alkaline-phosphatase and appropriate chromogens (Billingsley *et al.*, 1985). In order to demonstrate Ca^{2+} -dependence of binding, 5 mM-EGTA was used for certain blots instead of 1 mM- Ca^{2+} .

Uptake of [^{35}S]methionine and ^{32}P . Conidia ($1 \times 10^6 \text{ ml}^{-1}$) were incubated with shaking (75 r.p.m.) for up to 5 h at 27 °C in 50 ml YEM containing 20 μCi (740 kBq) carrier-free [^{32}P]phosphoric acid or [^{35}S]methionine in the presence or absence of R24571 or La^{3+} . At set intervals a 200 μl sample of each conidial suspension was spotted directly on to a glass-fibre (Whatman GF/C) disc. The conidia were washed (five $\times 20 \text{ cm}^3$) with 0.2 M-potassium phosphate buffer containing KCl (0.2 M) and Tween 80 (0.05%). The conidia were further washed in 95% ethanol (three $\times 10 \text{ cm}^3$), dried and counted with their filters in 6 ml of Cytosint (ICN) cocktail using a Beckman LS5801 scintillation counter. This determination represents the total uptake of [^{35}S]methionine or [^{32}P]orthophosphate by conidia.

^{32}P and ^{35}S incorporation into proteins. Conidia ($1 \times 10^6 \text{ ml}^{-1}$) or standard fungal inocula (1 g wet wt) from 32 h SDB cultures were incubated with shaking (75 r.p.m.) for up to 10 h at 27 °C in 10 ml YEM or chitin buffer media containing 0.2 mCi (7.4 MBq) carrier-free [^{32}P]phosphoric acid or [^{35}S]methionine in the presence or absence of drugs. Proteins secreted into growth media were precipitated by incubating overnight at -20 °C with 3 vols 0.1 M-ammonium acetate in methanol. Proteins were collected by centrifugation (10000 g, 10 min) and redissolved in water. Proteins were extracted from conidia or mycelia and prepared for electrophoresis as described below.

SDS-PAGE. Total cellular proteins were extracted from conidia or mycelia as previously described (St Leger *et al.*, 1989b) except that the extraction buffer was supplemented with the phosphatase inhibitors NaF (10 mM) and sodium molybdate (10 mM). Proteins were processed for and subjected to SDS-PAGE (Laemmli, 1970). Unless stated otherwise, acrylamide concentrations of 8 or 15% (w/v) were used to gain maximum resolution for molecular mass estimates of high and low molecular mass polypeptides, respectively. Radiolabelled proteins were detected by autoradiography (St Leger *et al.*, 1989b).

Plasma membrane preparation. Plasma membranes were isolated from mycelia from 32 h SDB cultures by β -glucuronidase digestion of the cell wall, gentle homogenization and differential centrifugation (Bowman & Bowman, 1988). Contamination by endoplasmic reticulum was removed by sucrose gradient centrifugation (Borgeson & Bowman, 1983). The membrane-associated ATPase, assayed by the method of Serrano (1978), was orthovanadate-sensitive, oligomycin-insensitive, indicating that the plasma membranes were free from mitochondrial contamination (Goffeau & Slayman, 1981). The absence of nucleic acids, extracted by the guanidium/hot phenol method (Maniatis *et al.*, 1982) and assayed at 260 nm, indicates that the membranes were free of cytoplasmic contamination.

Miscellaneous. Nuclei were stained using DAPI as described previously (St Leger *et al.*, 1989a). Chymoelastase activity (Pr1) was determined using succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide as substrate (St Leger *et al.*, 1987). Protein concentration was determined by the method of Bradford (1976). Ca^{2+} antagonists were added to cultures from stock solutions in distilled water [$\text{La}(\text{NO}_3)_3$, CaCl_2], ethanol (ionophores) or dimethylsulphoxide (CaM antagonists). The final concentration of ethanol or dimethylsulphoxide was $\leq 0.04\%$ which had no effect on protein synthesis or phosphorylation in controls.

RESULTS

Detection of CaM and CaM-binding proteins

CaM levels in ungerminated conidia, germlings (13 h YEM cultures) and mycelia (32 h SDB cultures) were 4.6 ± 1.05 , 2.9 ± 0.75 and $4.1 \pm 0.61 \mu\text{g}$ rat equivalent CaM (mg total protein) $^{-1}$ respectively (n = five independent measurements).

Six prominent CaM-binding proteins (37.5, 42, 44, 69.5, 78 and 84 kDa) were detected in conidia and germlings (Fig. 1). Binding of biotinylated CaM was prevented by replacing Ca^{2+} with EGTA, indicating the Ca^{2+} dependence of these interactions. The exception was the 37.5 kDa protein which showed low binding with EGTA. Fifteen relatively minor Ca^{2+} -dependent CaM-binding proteins (18–35 kDa) were also detected.

Effect of CaM and Ca^{2+} antagonists on germination

A range of structurally diverse agents known to act as antagonists of CaM function were potent inhibitors of germination of conidia in YEM. The rank order of effectiveness of the CaM antagonists in terms of IC_{50} values (μM) (the concentration of inhibitor reducing germination by 50%) and the binding affinity of the antagonists for CaM [K_i (μM) values from Asano *et al.*, 1985] were: compound 48/80, 1.1 (K_i 0.3); R24571, 1.4 (K_i 0.3); trifluoperazine, 14 (K_i 1); W-7, 23 (K_i 11) and W-5, > 200 (K_i 90). This correlation between effectiveness and CaM-binding affinity is convincing evidence for the specificity of action of the antagonists (Roufogalis *et al.*, 1982; Asano & Stull, 1985). Those conidia which did germinate produced short, broad, frequently asymmetric germ-tubes. Examination of affected germlings ($1.4 \mu\text{g ml}^{-1}$ of the CaM

Fig. 1. Binding of biotinylated CaM to CaM-binding proteins. Proteins (100 μ g) from conidia (1) and germlings (2) were subjected to SDS-PAGE (13%, w/v, acrylamide), electroblotted on to nitrocellulose, incubated with biotinylated CaM in the presence of Ca^{2+} , and the blots developed with avidin-alkaline-phosphatase. In incubation mixtures containing 5 mM-EGTA, little binding of biotinylated CaM was detected (control, germling proteins).

antagonist R24571) after staining with DAPI showed that nuclear division had not occurred. However, 43% of conidia had swollen from $4 \times 2.5 \mu\text{m}$ to about $8 \times 3.5 \mu\text{m}$, an event that precedes normal germination (Dillon & Charnley, 1989). Increasing the concentration of R24571 ($7 \mu\text{g ml}^{-1}$) further repressed the frequency of spore swelling ($<10\%$).

Incorporation into growth media (YEM) of the Ca^{2+} chelator EGTA or plasmalemma Ca^{2+} -channel blockers (nifedipine or verapamil) had no effect on germination, indicating that this process does not require an influx of Ca^{2+} . However, without extracellular Ca^{2+} (3 mM-EGTA) germ-tube growth was limited to 85–130 μm as compared with $>400 \mu\text{m}$ after 30 h in unmodified YEM.

Table 1. *Effect of Ca²⁺ antagonists and ionophores on germination and germ-tube growth*

Conidia were incubated for 14 h with inhibitor in YEM, pH 6.8.

Treatment	Germination (%) (<i>n</i> = 300)	Mean germ-tube growth (μm) (± SE, <i>n</i> = 100)
None	84	17 ± 6.3
CaCl ₂ (1 mM)	85	19 ± 7.1
EDTA (5 mM)	82	16 ± 4.2
A23187 (2 μM)	72	12 ± 6.3
A23187 (2 μM) + CaCl ₂ (1 mM)	87	28 ± 6.7
A23187 (2 μM) + EGTA (5 mM)	65	8 ± 2.3
A23187 (6 μM)	54	7 ± 0.4
A23187 (6 μM) + EGTA (5 mM)	8	6 ± 2.0
A23187 (10 μM) + Ca ²⁺ (1 mM)	75	14 ± 2.5
Br-A23187 (5 μM)	81	13 ± 3.6
Br-A23187 (5 μM) + CaCl ₂ (1 mM)	83	27 ± 8.3
Br-A23187 (5 μM) + EGTA (5 mM)	62	10 ± 4.0
Br-A23187 (10 μM) + EGTA (5 mM)	12	8 ± 0.3
La(NO ₃) ₃ (10 μM)	73	15 ± 3.2
(25 μM)	54	9 ± 1.4
(50 μM)	2	5
Fluorescein isothiocyanate (150 μM)	80	16 ± 6.8
Ryanodine (50 μM)	81	18 ± 5.4

Ionophoresis experiments were done using the divalent cation ionophore A23187 or the Ca²⁺-selective ionophore Br-A23187 (Deber *et al.*, 1985). In the presence of Ca²⁺, high concentrations of ionophore did not affect germination frequency. Depleting intracellular Ca²⁺ levels (A23187 + EGTA or Br-A23187 + EGTA) reduced germination by an extent dependent on the concentration of the ionophore (Table 1). A similar effect was produced by low concentrations of the Ca²⁺ antagonist La³⁺ (Anghileri, 1987). At higher concentrations La³⁺ (> 50 μM) was a potent inhibitor of germination (Table 1). The inhibitory effects of R24571 or La³⁺ on germination were not reduced by extending the duration of incubation up to 4 d. Specific antagonists of Ca²⁺ transport across endoplasmic reticulum (ryanodine and fluorescein isothiocyanate) did not affect germination frequency.

Effect of Ca²⁺ antagonists on the uptake of [³²P]orthophosphate and [³⁵S]methionine and their incorporation into proteins during germination

When conidia were incubated in YEM for 10 h (70% germination frequency) in the presence of [³⁵S]methionine or [³²P]phosphoric acid, 60 and 32 radiolabelled proteins were detected respectively (Fig. 2). Most of the newly made proteins detected using [³⁵S]methionine as precursor could also be identified in ungerminated conidia using Coomassie blue stain (Fig. 2). Some of the major [³⁵S]methionine- and ³²P-labelled polypeptides possessed the same molecular masses; amongst the more prominent were bands at 16, 19.5, 20.5, 21.5, 25.5, 33, 39 (Fig. 2), 79.2 and 98.4 kDa [estimated from an SDS-PAGE gel (8% w/v, acrylamide); data not shown]. Compound R24571 (2.8 μg ml⁻¹) and La³⁺ (50 μM) repressed uptake of [³⁵S]methionine over 5 h by 91% (2.6 ± 0.5 c.p.m. × 10⁻³) and 89% (3.3 ± 0.3 c.p.m. × 10⁻³) respectively compared with controls (28.8 ± 2.5 c.p.m. × 10⁻³; results expressed as $\bar{X} \pm \text{SD}$, *n* = 3). Likewise, compound R24571 and La³⁺ repressed uptake of [³²P]orthophosphate by 95% (0.5 ± 0.1 c.p.m. × 10⁻³) and 87% (1.3 ± 0.1 c.p.m. × 10⁻³) respectively (controls 10 ± 0.8 c.p.m. × 10⁻³; $\bar{X} \pm \text{SD}$, *n* = 3). Similar repression was obtained by depleting intracellular Ca²⁺ (Br-A23187 (10 μM) + EGTA (5 mM). SDS-PAGE analysis confirmed that R24571 (2.8 μg ml⁻¹), La³⁺ (50 μM) and ionophoresis (EGTA + Br-A23187) entirely prevented incorporation of [³²P]orthophosphate (Fig. 3) or [³⁵S]methionine into proteins (data not shown). None of the proteins stained by Coomassie blue were selectively degraded during treatment of conidia with R24571 (Fig. 2, lane 2). Other treatments which might perturb intracellular Ca²⁺ gradients, e.g. Br-A23187 + Ca²⁺ or H-7 [1-(5-isouquinolinesulphonyl)-2-methylpiperazine dihydrochloride, the H-series inhibitor

Fig. 2. SDS-PAGE (13%, w/v, acrylamide) analysis of newly synthesized proteins during germination of conidia of *M. anisopliae*. 1, Proteins (80 µg) from ungerminated conidia stained with Coomassie blue dye. 2, Stained proteins (80 µg) from conidia incubated (10 h) in YEM containing R24571 (2 µM). 3, 4, 5, 6, Autoradiographs of incorporation of [³⁵S]methionine (3,4) or ³²P (5,6) into proteins (30 µg) from conidia incubated for 10 h at 27 °C in YEM. Autoradiographs were incubated for 12 h (4,6) or 24 h (3,5).

with highest affinity for protein kinase C (Hidaka & Tanaka, 1987)] did not affect total uptake of [³²P]orthophosphate but selectively repressed phosphorylation at 15.7, 36 and 38 kDa. In addition, H-7 repressed phosphorylation at 27 kDa (Fig. 3). These effects were not correlated with a change in the profile of protein synthesis.

Effect of Ca²⁺ antagonists on protein synthesis and extracellular production of Pr1

Extracellular Pr1 is formed *de novo* within 3 h of mycelia being transferred from SDB (catabolite repression; Pr1 not produced) to a medium containing chitin (Table 2). Production of extracellular Pr1 was unaffected by the presence of H-7, H-8 [*N*-(2-methylamino)ethyl-5-isoquinolinesulphonamide dihydrochloride, the H-series inhibitor with highest affinity for cyclic-nucleotide-dependent kinases (Hidaka & Tanaka, 1987)], nifedipine and EGTA. Antagonists of CaM (compound 48/80 and R24571) were also without effect, even at concentrations tenfold

Fig. 3. Effects of Ca^{2+} antagonists on protein phosphorylation in germinating conidia of *M. anisopliae*. Autoradiographs of ^{32}P incorporation into proteins of conidia incubated for 10 h at 27°C in (1) unmodified YEM; (2) YEM containing H-7 ($50\text{ }\mu\text{M}$); (3) Br-A23187 ($10\text{ }\mu\text{M}$) + CaCl_2 (1 mM); (4) Br-A23187 ($10\text{ }\mu\text{M}$) + EGTA (5 mM); (5) $\text{La}(\text{NO}_3)_3$ ($50\text{ }\mu\text{M}$); (6) R24571 ($2\text{ }\mu\text{M}$). Proteins ($30\text{ }\mu\text{g}$) were subjected to SDS-PAGE (13% , w/v, acrylamide) and autoradiographed.

Table 2. Effect of Ca^{2+} antagonists on protease production by *M. anisopliae* after 3 h in medium containing chitin (0.2%)

Values are means of three replicates $\pm$ SD. The results are representative of two similar experiments.

Inhibitor*	Pr1 production (nmol <i>p</i> -nitroanilide hydrolysed $\text{ml}^{-1}\text{ min}^{-1}$)
None	13.2 ± 1.23
EGTA (5 mM)	12.8 ± 1.28
Br-A23187 ($6\text{ }\mu\text{M}$)	10.4 ± 1.84
Br-A23187 ($6\text{ }\mu\text{M}$) + EGTA (5 mM)	0.3 ± 0
Br-A23187 ($6\text{ }\mu\text{M}$) + Ca^{2+} (1 mM)	9.5 ± 1.55
$\text{La}(\text{NO}_3)_3$ ($50\text{ }\mu\text{M}$)	2.3 ± 0.2
R24571 ($10\text{ }\mu\text{g ml}^{-1}$)	13.0 ± 0.6
Compound 48/80 ($10\text{ }\mu\text{g ml}^{-1}$)	11.6 ± 1.3

* Nifedipine, H-7 and H-8 were also tested and found to be without effect on Pr1 synthesis.

Fig. 4. Effects of Ca^{2+} antagonists on protein synthesis and secretion by mycelia of *M. anisopliae*. Autoradiograph of [^{35}S]methionine incorporation during protein synthesis (1–3) and secretion (4–6) by mycelia incubated for 5 h at 27 °C in (1,4) unmodified chitin (0.2%) media; (3,5) chitin media supplemented with Br-A23187 (10 μM) + EGTA (5 mM); (2,6) R24571 (2 μM). Proteins (30 μg) were subjected to SDS-PAGE and autoradiographed.

higher than those required to inhibit germination (Table 2). In contrast, levels of extracellular Pr1 were greatly reduced by depleting or antagonizing intracellular Ca^{2+} using Br-A23187 + EGTA or La^{3+} , respectively.

In spite of the effect of Ca^{2+} depletion on extracellular levels of Pr1, the profile of protein synthesis in mycelia determined after incubation with [^{35}S]methionine was almost unaffected by Br-A23187 + EGTA or R24571 (Fig. 4). However, Ca^{2+} depletion reduced extracellular release of several high molecular mass proteins and a 25 kDa band previously shown to be Pr1 by Western blot analysis (St Leger *et al.*, 1989b), while increasing the release of lower molecular mass polypeptides (Fig. 4). This was not due to a toxic effect by Br-A23187 or EGTA as these agents did not affect the profile of protein secretion when applied separately.

Effect of Ca^{2+} antagonists on phosphorylation of proteins during Pr1 synthesis

Incubation of mycelia for 1 h in chitin media allowed phosphorylation of at least 40 proteins (Fig. 5). Treating mycelia with Br-A23187 + EGTA or La^{3+} produced sharp decreases in net phosphorylation (Fig. 5 and data not shown). Elevating cytoplasmic Ca^{2+} with a combination of

Fig. 5. Effects of Ca^{2+} antagonists on protein phosphorylation in mycelia of *M. anisopliae*. Autoradiograph of ^{32}P incorporation into proteins of mycelia incubated for 1 h at 27°C in (1) unmodified chitin (0.2%) media; (2) chitin media containing H-7 (50 μM); (3) R24571 (2 μM); (4) Br-A23187 (10 μM) + EGTA (5 mM) + R24571 (2 μM); (5) Br-A23187 (10 μM); (6) Br-A23187 (10 μM) + CaCl_2 (1 mM) + H-7 (50 μM); (7) Br-A23187 (10 μM) + CaCl_2 (1 mM) + R24571 (2 μM). Proteins (30 μg) were subjected to SDS-PAGE (13%, w/v, acrylamide) and autoradiography.

Br-A23187 + Ca^{2+} (1 mM) did not affect phosphorylation. In spite of repressing Pr1 production, addition of nutrients (1% alanine + 1% glucose) had no detectable effect on the phosphorylation profile (data not shown).

CaM antagonists (R24571) increased [^{32}P]orthophosphate incorporation into 17 and 33 kDa proteins (Fig. 5) as did compound 48/80 (data not shown). This effect was not inhibited by cycloheximide indicating that *de novo* synthesis of substrate is not required. However, Ca^{2+} depletion (Br-A23187 + EGTA) over-rode the enhancing effect of CaM antagonists indicating that radiolabelling is Ca^{2+} dependent.

The involvement of the C-kinase pathway was tested with the C-kinase activator TPA and the inhibitor H-7 (Hidaka & Tanaka, 1987). While TPA had no effect (data not shown), H-7 at high concentrations (>100 μM) repressed phosphorylation of a 27 kDa protein (Fig. 5). A similar effect was produced by H-8 (inhibitor of cyclic-nucleotide-dependent kinase) so the form of control of the responsible kinase is in doubt.

Phosphoamino acid analysis

Proteins from mycelia preincubated with [^{32}P]phosphoric acid were analysed by SDS-PAGE and the gel incubated in 1 M-NaOH at 55°C for 1 h prior to autoradiography. This eliminates phosphate present on serine and threonine residues but not on tyrosine residues (Cooper &

Fig. 6. Identification of tyrosine phosphorylated proteins in (1) total protein (100 µg) extract from mycelia; (2) proteins solubilized from plasma membranes by 10 mM-Tris/HCl (pH 7.5) supplemented with 0.1% Triton X-100; (3) 10 mM-Tris/HCl (pH 7.5) alone. Proteins were subjected to SDS-PAGE (13%, w/v, acrylamide), electroblotted on to nitrocellulose, probed with anti-PTYR-bodies and ^{125}I -protein A and autoradiographed.

Hunter, 1981). Most of the radiolabel was removed by this treatment and the few remaining bands were almost indistinct against a high background (data not shown). Therefore, most of the proteins are phosphorylated at serine and threonine residues.

Immunoblotting total cellular proteins with anti-PTYR (Wang, 1988) revealed prominent bands at 38 and 34.5 kDa, and minor bands of higher molecular mass (Fig. 6). The predominant 38 kDa protein could also be identified on isolated plasma membranes. Partial disruption of the membrane structure by preincubation with Triton X-100 solubilized more of the 38 kDa protein than washing with buffer alone (Fig. 6). Phosphorylation at tyrosine was unaffected by growing the mycelia in a nutrient rich medium (0.2% chitin, 1% alanine and 1% glucose) versus chitin (0.2%) alone or preincubation of mycelia (5 h) with Br-A23187 + CaCl_2 or EGTA, R24571, H-7, H-8 or cAMP.

DISCUSSION

Germination of conidia of *M. anisopliae* requires uptake of nutrients and involves *de novo* synthesis of protein (Dillon & Charnley, 1989; St Leger *et al.*, 1989a). Uptake of [^{35}S]methionine or [^{32}P]orthophosphate into conidia and their incorporation into proteins during germination fulfill essential criteria for Ca^{2+} /CaM-dependent processes (Cheung, 1980). Thus CaM and CaM target-proteins were detected in ungerminated conidia. Also, depleting intracellular Ca^{2+} , or treatment with CaM antagonists massively reduced uptake of both isotopes, indicative of a non-specific inhibition of nutrient uptake. By contrast, inhibitors of C-kinase and cyclic-

nucleotide-dependent kinases affected individual phosphorylation events without altering net phosphorylation, protein synthesis and germination.

The presence of CaM and the same CaM target-proteins in both ungerminated conidia and germlings indicates that the machinery for CaM activation of germination-related processes exists preformed before germination and therefore does not require *de novo* protein synthesis. CaM levels in conidia and mycelia were similar (about 0.3% of total proteins assuming a quantitatively similar response being given by CaM from *M. anisopliae* and from rat) indicating little if any increase in CaM synthesis relative to the total protein during germination and growth. This is in contrast to higher plants where CaM levels fluctuate markedly during growth and differentiation (Cocucci & Negrini, 1988). Furthermore, the number and concentration of CaM-binding proteins was similar in conidia and germlings. Work on mammalian systems has demonstrated a close relationship between CaM concentrations and those of its target proteins which enables CaM to modulate the effect of the Ca^{2+} signal (Klee & Newton, 1985).

As CaM is activated by Ca^{2+} , its effect on germination events may be linked with mobilization of intracellular Ca^{2+} reserves. Consistent with this, depletion of intracellular Ca^{2+} or treatment with La^{3+} duplicated the effects of R24571. Inhibition by La^{3+} but not by fluorescein isothiocyanate or ryanodine implies that the Ca^{2+} is derived from mitochondrial stores rather than the endoplasmic reticulum (Reid & Bygraves, 1974; Mitchinson *et al.*, 1982; Nagasaki & Fleischer, 1988). This is consistent with the known ultrastructure of *Metarhizium* conidia which contain large mitochondria but little endoplasmic reticulum (Dillon & Charnley, 1989). However, La^{3+} can compete with Ca^{2+} for many binding sites including those on the surface of the cell and it may affect physiology in different ways (Anghileri, 1987).

A precise knowledge of how CaM functions in initiating germination awaits identification of the CaM-binding proteins. That several CaM-binding proteins were detected in conidia and germlings is strong evidence that CaM is a multifunctional regulator. However, the massive repression of [^{32}P]orthophosphate uptake and phosphorylation both by Ca^{2+} depletion and treatment with R24571 is indicative of action by Ca^{2+} /CaM-dependent protein kinase(s), the primary mechanism of CaM action in other systems (Nairn *et al.*, 1985). Similarities in overall protein composition between conidia and germlings (St Leger *et al.*, 1989b) and the fact that most proteins identified by Coomassie blue staining were also detectable using [^{35}S]methionine as a precursor suggests that kinase activity would not require *de novo* synthesis of protein substrates. As germination and protein synthesis can take place without nuclear division and in the presence of hydroxyurea, an inhibitor of DNA synthesis (St Leger *et al.*, 1989a), inhibition of nuclear division by CaM antagonists is unlikely to be involved in repression of protein synthesis.

CaM antagonists do not inhibit incorporation of isotopes into proteins, and extracellular secretion of proteins by mycelia over a 3 h period. Thus the continuous activity of CaM may not be required after protein synthesis is initiated. Furthermore, initiation of the synthesis of Pr1 is not affected by CaM inhibitors. Therefore CaM is not involved in the regulation of at least one protein produced post-germination. The effect of CaM antagonists in selectively enhancing ^{32}P incorporation into 17 and 33 kDa polypeptides suggests the existence of a CaM-stimulated phosphatase as reported in vertebrates (Nairn *et al.*, 1985).

The lack of inhibition of net phosphorylation in mycelia by CaM antagonists or the C-kinase inhibitor H-7 indicates that the repression of phosphorylation produced by Ca^{2+} depletion or antagonism (La^{3+} addition) may have resulted directly from Ca^{2+} perturbation. Ca^{2+} -dependent, CaM-independent kinases are known in plants (Hetherington & Trewavas, 1984) and several proteins besides C-kinase and CaM can mediate the effect of Ca^{2+} in different systems (Carafoli, 1987). Ca^{2+} depletion did not prevent protein synthesis so the effect on phosphorylation may be quite specific rather than due to gross disruption of membrane processes dependent on Ca^{2+} gradients.

We were unable to correlate Pr1 production with a single phosphorylation event. Catabolite repression of Pr1 synthesis had little effect on the phosphorylation profile, while Ca^{2+} depletion and La^{3+} repressed both Pr1 secretion and net phosphorylation. In many systems, Ca^{2+} plays an important role in enzyme release by exocytosis (Knight, 1986).

Immunoblot detection of tyrosine phosphorylation revealed two major bands (38 and 34.5 kDa). Compared to the ^{32}P autoradiographs, these phosphoproteins do not belong to the major phosphorylated proteins which are all alkali susceptible. Thus phosphotyrosine is a scarce residue in *M. anisopliae*, as well as some other systems (Hirsch-Behnam & Barnekow, 1988). Phosphotyrosine has been detected in yeast proteins and a plasma-membrane preparation was shown to produce alkali-resistant phosphorylation of casein (Castellanos & Mazon, 1985). Similarly, a component of the major tyrosine phosphoprotein is localized on the plasma membrane. This is significant as tyrosine kinases catalyse autophosphorylation and are recognized by anti-PTYR antibody (Pilch, 1989). Tyrosine kinase in vertebrate systems is often intrinsic to surface receptors, ligand-receptor interactions leading directly to protein phosphorylation of tyrosine without the requirement for intermediary messengers (McNeil & Taylor, 1987). These phosphorylation events are consequently unresponsive to Ca^{2+} and cAMP as reported here for *M. anisopliae*. By analogy tyrosine-specific protein kinases could themselves be important mediators of primary messages in *M. anisopliae*.

The data on Ca^{2+} -promoted protein phosphorylation indicates that Ca^{2+} has an important role in regulating phosphorylation at threonine and serine residues. Furthermore, CaM acts as a Ca^{2+} effector in conidia where its activity is crucial for protein synthesis and germination. While there is strong evidence for pathways for the transduction of stimuli in this fungus that involve Ca^{2+} and CaM similar to those known in plant and animal systems, additional research will be required to elucidate the nature of the protein kinases and their substrates to understand how Ca^{2+} fluxes activate cell metabolism.

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