cAMP alteration of growth rate of *Aspergillus fumigatus* and *Aspergillus niger* is carbon-source dependent

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cAMP signalling has been shown to be essential for normal growth, morphology and virulence in fungal pathogens of both plants and animals. The effects of exogenous cAMP on the growth of the opportunistic pathogen *Aspergillus fumigatus* were compared to those of *Aspergillus niger*, which has previously been demonstrated to respond to extracellular cAMP. Both cAMP and phosphodiesterase inhibitors markedly reduced the radial growth rate of *A. niger* after 48 h on minimal medium with glucose as the carbon source, whereas the growth of *A. fumigatus* was not affected by cAMP. However, when glycerol, which does not initiate carbon catabolite repression, was used as a carbon source, cAMP inhibited the radial growth rate of only *A. fumigatus* ($P < 0.05$). The addition of cAMP to glycerol-minimal medium resulted in a fourfold increase in protein kinase A activity in *A. fumigatus* cell extracts when compared with pre-treatment samples. The protein kinase A activity in *A. fumigatus* cell extracts from cultures grown in glucose did not change significantly with the addition of cAMP. These studies demonstrate that although the growth rates of both *A. fumigatus* and *A. niger* are sensitive to the addition of exogenous cAMP, the response of each organism is distinct and dependent on the carbon source used.

Keywords: PKA, phosphodiesterase inhibitor, glucose repression

INTRODUCTION

The ability of organisms to respond to extracellular stimuli is essential for survival. The cAMP/protein kinase A (PKA) signalling pathway has been demonstrated to be critical in detecting environmental changes and translating those changes into a biological response in many organisms (Thevelein, 1994). Upon receptor activation, a receptor-coupled G-protein activates adenylyl cyclase. Adenylyl cyclase catalyses the conversion of ATP to cAMP, which accumulates in the cell, leading to activation of PKA (Lengeler *et al.*, 2000). PKA is a heterotetrameric molecule composed of two regulatory subunits and two catalytic subunits. cAMP binds to the regulatory subunits, which causes a conformational change that releases the catalytic subunits to autophosphorylate one another and to phosphorylate downstream targets (Taylor & Radzio-Andzelm, 1994). The cAMP/PKA pathway has been demonstrated to play an

important role in response to environmental stress, particularly nutritional stress, in the model organism *Saccharomyces cerevisiae*.

The cAMP/PKA pathway of *S. cerevisiae* has been proposed as the mechanism by which the cell detects changes in glucose availability and adjusts its metabolic machinery accordingly. To maintain metabolic efficiency, the presence of glucose in the medium stimulates the PKA pathway, which leads to repression of genes involved in metabolizing non-glucose carbon sources (Thevelein, 1994). Cells with increased PKA activity, due to deletion of the PKA regulatory subunit, exhibit an inability to grow on non-fermentable, non-repressing carbon sources such as glycerol and ethanol, as well as the weakly fermentable, weakly repressing carbon source galactose (Toda *et al.*, 1987). These mutants also fail to sporulate in response to nutritional stress (Malone, 1990). Mutants with reduced PKA activity express genes normally associated with growth on non-glucose carbon sources, and they sporulate on rich medium, which usually suppresses sporulation in wild-type cells (Casperson *et al.*, 1985). The effects of PKA

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; PKA, protein kinase A.

signalling have also been studied in *Aspergillus* species. In *Aspergillus niger*, strains that overexpress the catalytic subunit of PKA exhibit a slower growth rate relative to wild-type cells (Bencina *et al.*, 1997). They display a similar phenotype to that of wild-type *A. niger* grown on glucose-containing medium supplemented with cAMP (Bencina *et al.*, 1997). However, strains of *Aspergillus nidulans* that overexpress the catalytic subunit of PKA exhibit no alterations with regard to growth rate when grown on medium containing glucose (Shimizu & Keller, 2001). Although features of PKA signalling have been reported in these two *Aspergillus* species, the PKA pathway in *Aspergillus fumigatus* has not been studied.

A. fumigatus is an opportunistic pathogen of humans that plays a key role in the compost cycle in nature. To grow in the lung, the organism must be able to sense the change in environment and translate that change into an appropriate metabolic response. It has recently been shown that transcript levels of the PKA regulatory subunit are elevated when the fungus grows in the presence of endothelial cells, suggesting that PKA may play a role in signalling that environmental change (Rhodes *et al.*, 2001). This would be compatible with data from the fungal plant pathogens *Ustilago maydis*, *Magnaporthe grisea* and *Colletotrichum trifolii*, as well as the human pathogens *Cryptococcus neoformans* and *Candida albicans*, in which PKA signalling has been shown to be required for wild-type virulence (D'Souza *et al.*, 2001; D'Souza & Heitman, 2001; Kronstad *et al.*, 1998; Kruger *et al.*, 1998; Sonneborn *et al.*, 1999; Xu *et al.*, 1997). Because little is known of the importance of this pathway and the effects it may have on the growth of *A. fumigatus*, the response of the organism to agonists and antagonists of the cAMP/PKA pathway was studied on media containing different carbon sources.

METHODS

Isolates and culture conditions. *A. fumigatus* H237 and *A. niger* isolate AS-3 are both clinical isolates. Isolates were maintained on Sabouraud's dextrose agar at 37 °C.

Radial growth assay. Conidia were harvested with sterile distilled water, filtered through Miracloth (Calbiochem) and counted in a haemocytometer. Ten microlitres of a conidial suspension (10^3 conidia μl^{-1}) were spotted onto *Aspergillus* minimal medium with 10 mM sodium nitrate as the nitrogen source (ASP MM) (Cove, 1966) plates or on to ASP MM plates containing cAMP, cAMP analogues or phosphodiesterase (PDE) inhibitors. Triplicate plates of all media were inoculated. Indicated carbon sources were used at 1% final concentrations. cAMP, cAMP analogues and the PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX) and theophylline were used at concentrations between 1 and 10 mM. Vehicle controls were performed with the appropriate amount of the respective solvent. Dose-response studies were used to choose suboptimal levels of cAMP and IBMX for experiments in which the compounds were combined to test for additive activity. All chemicals were obtained from Sigma.

Radial colony growth was monitored for cultures of *A. fumigatus* and *A. niger* at 37 °C every 12 h for 48 to 60 h.

Colony diameter was measured at each time point with callipers. Growth rates, in $\mu\text{m h}^{-1}$, were based on colony diameters measured at 24 and 48 h, except for acetate, which used measurements taken at 48 and 60 h. All experiments were performed a minimum of two times, and results from representative experiments are presented.

Enzyme activity assays. Fifty millilitre aliquots of ASP MM + 1% glucose were inoculated with 1×10^8 conidia and cultures were incubated at 37 °C, 225 r.p.m., for 12 h. Because glycerol does not support robust germination, conidia were allowed to germinate in ASP MM + 1% glucose for 6 h before being washed twice with ASP MM + 1% glycerol and transferred to 50 ml ASP MM + 1% glycerol. These cultures were then grown at 37 °C, 225 r.p.m. for 12 h. At the end of the growth period, hyphae were treated with cAMP at a final concentration of 10 mM and collected by vacuum filtration after 0, 5, 15 or 30 min continued incubation. Hyphae were washed quickly with ice-cold 0.8% NaCl, frozen in liquid nitrogen, and crushed with a mortar and pestle to obtain cell extracts. Samples were then resuspended in extraction buffer (25 mM Tris/HCl pH 7.4, 10 mM MgCl_2 , 150 mM NaCl, 1 mM DTT, 1 mM EDTA) and incubated on ice for 30 min. The homogenate was centrifuged at 10000 g for 10 min at 4 °C, and the supernatant was used in the assay. PKA enzyme activity was measured by kemptide phosphorylation (colorimetric PKA assay, Spinzyme format; Pierce) using the manufacturer's protocol with the following modification: no activator solution (500 μM cAMP) was used in the assay to measure the amount of PKA activity in the extracts. Purified bovine cardiac PKA-C (Pierce) was used as a standard. The amount of phosphorylated product was measured spectrophotometrically at 570 nm in a 96-well plate and normalized to the amount of total protein used in the assay. Protein was measured using the BCA assay (Pierce) as described by the manufacturer. Triplicate cultures were processed for cell extracts under each experimental condition. Each experiment was repeated and results from representative experiments are shown.

Statistical analysis. Statistical significance was determined by one tailed, paired *t*-test for means with a 95% confidence interval. Values are expressed as the mean $\pm$ SEM. Statistical tests were performed using Microsoft Excel.

RESULTS AND DISCUSSION

The cAMP/PKA pathway has been demonstrated to be a critical mediator of growth and response to nutritional stress in *S. cerevisiae* (Thevelein & de Winde, 1999). Proper pathway function is also required for virulence in the plant pathogens *M. grisea* and *U. maydis*, as well as in the mammalian pathogens *Can. albicans* and *Cry. neoformans* (Lengeler *et al.*, 2000). Despite the importance of the cAMP/PKA pathway in the growth, stress response and virulence of fungi, little is known of the function of this pathway in the opportunistic pathogen *A. fumigatus*.

Previous studies in *A. niger* demonstrated a reduction in growth when glucose-containing medium was supplemented with cAMP. Likewise, strains of *A. niger* that overexpress the catalytic subunit of PKA also show growth inhibition (Bencina *et al.*, 1997). To determine whether PKA signalling plays a role in *A. fumigatus* growth, colonies of *A. fumigatus* were grown on

Table 1. Growth rate ($\mu\text{m h}^{-1}$) of *A. fumigatus* and *A. niger* when grown on minimal medium with various carbon sources in the presence of cAMP, cAMP analogues and PDE inhibitors

Results were not statistically significant ($P > 0.05$) unless otherwise indicated.

Medium	Growth rate	
	<i>A. fumigatus</i>	<i>A. niger</i>
ASP MM + glucose		
Alone	712 $\pm$ 15	694 $\pm$ 3
+ 10 mM cAMP	721 $\pm$ 10	128 $\pm$ 6*
+ 10 mM Dibutyl- <i>l</i> -cAMP	762 $\pm$ 15	547 $\pm$ 11†
+ Ethanol‡	731 $\pm$ 7	562 $\pm$ 4
+ 10 mM 8-Bromo-cAMP	750 $\pm$ 1	379 $\pm$ 4†
+ 1 mM DMSO§	707 $\pm$ 11	479 $\pm$ 5
+ 1 mM IBMX	632 $\pm$ 10†	414 $\pm$ 4*
+ 10 mM DMSO§	586 $\pm$ 1	315 $\pm$ 4
+ 10 mM IBMX	207 $\pm$ 5*	243 $\pm$ 14†
+ NaOH	701 $\pm$ 6	567 $\pm$ 13
+ 10 mM Theophylline	236 $\pm$ 1*	480 $\pm$ 4*
ASP MM + glycerol		
Alone	708 $\pm$ 6	418 $\pm$ 6
+ 10 mM cAMP	656 $\pm$ 12†	401 $\pm$ 6
+ 1 mM DMSO§	700 $\pm$ 17	386 $\pm$ 4
+ 1 mM IBMX	615 $\pm$ 15*	332 $\pm$ 6*
+ 10 mM DMSO§	492 $\pm$ 9	279 $\pm$ 17
+ 10 mM IBMX	206 $\pm$ 1*	150 $\pm$ 15*
ASP MM + acetate		
Alone	386 $\pm$ 22	475 $\pm$ 13
+ 10 mM cAMP	281 $\pm$ 20†	407 $\pm$ 11†
+ 0.5 mM IBMX	369 $\pm$ 19	475 $\pm$ 21
ASP MM + galactose		
Alone	560 $\pm$ 24	INS¶
+ 10 mM cAMP	470 $\pm$ 12†	INS¶
+ 0.5 mM IBMX	417 $\pm$ 12†	INS¶

* $P < 0.001$.

† $P < 0.05$.

‡ Growth control for 8-bromo-cAMP.

§ Growth control for IBMX.

|| Growth control for theophylline.

¶ Insufficient growth in 60 h.

minimal medium with different carbon sources, with or without cAMP.

Effects of exogenous cAMP

A. fumigatus and *A. niger* were grown on glucose-containing medium supplemented with cAMP to determine the effect of cAMP on growth rate. Although *A. niger* displayed a reduction in growth rate ($P < 0.001$) on medium containing cAMP, as previously demonstrated (Bencina *et al.*, 1997), there was no growth rate reduction seen with *A. fumigatus* (Table 1; Fig. 1). To test the possibility that *A. fumigatus* was impermeable to cAMP

or was degrading the cAMP, the non-hydrolysable and lipid-soluble cAMP analogues 8-bromo-cAMP and dibutyl-*l*-cAMP were used in place of cAMP. Neither cAMP analogue changed the growth rate of *A. fumigatus*, but the growth rate of *A. niger* was significantly reduced by both analogues (Table 1). The use of PDE inhibitors with intact cells has been reported to result in an increase in intracellular cAMP levels (Beebe & Corbin, 1986). The addition of either of the PDE inhibitors IBMX or theophylline to the medium led to a reduction in the growth rate of both *A. fumigatus* and *A. niger*, suggesting either that cAMP and its analogues were not able to enter the cells of *A. fumigatus* or that

Fig. 1. Growth of *A. niger* and *A. fumigatus* after 48 h at 37 °C on ASP MM containing glucose, with and without 10 mM cAMP. A. *A. fumigatus*, ASP MM. B. *A. fumigatus*, ASP MM + 10 mM cAMP. C. *A. niger*, ASP MM. D. *A. niger* ASP MM + 10 mM cAMP.

the inhibitors were decreasing growth through another, non-PKA related mechanism. To demonstrate that the decreases in growth rate obtained with cAMP and PDE inhibitors were due to modulation of the cAMP/PKA pathway, rather than to non-specific toxicity, suboptimal concentrations of cAMP and IBMX were used in combination to show an additive effect on growth inhibition (Beebe & Corbin, 1986). Indeed, on minimal medium plus glucose, the growth rate of *A. niger* was significantly inhibited by the combination ($P < 0.05$) when compared with the growth rate on cAMP alone and IBMX alone (Table 2). In contrast, *A. fumigatus* did not display a significant change in growth rate on medium containing cAMP and IBMX when compared with both alone. Results with theophylline were similar to those obtained with IBMX (data not shown). These results support the interpretation that the decreased growth rate of *A. fumigatus* when grown in the presence of PDE inhibitors is due to toxicity or some other non-PKA related mechanism, whereas a PKA-specific mechanism appears to be active in *A. niger*.

Two lines of reasoning led us to investigate the role that carbon source might play on the growth response of *A. fumigatus* to cAMP. In *S. cerevisiae*, glucose is believed to act through a G-protein coupled receptor, Gpr1p, to cause a Gpa2p-dependent, transient increase in intracellular cAMP, followed by a longer-lived increase mediated through intracellular acidification-triggered RAS activation of adenyl cyclase (Kraakman *et al.*, 1999). In *A. niger* growing on sucrose, intracellular acidification is also believed to lead to increased cAMP synthesis (Gradišnik-Grapulín & Legiša, 1997). In both cases, the result is that the cAMP/PKA pathway is activated. Therefore, fructose, galactose, glycerol and acetate were chosen for testing, since they represent carbon sources that vary in their ability to be fermented and to participate in carbon catabolite repression. Fructose is a weakly repressing, fermentable carbon source; galactose is non-repressing and weakly fermentable; and glycerol and acetate are non-repressing and non-fermentable. When both *A. niger* and *A. fumigatus* were grown on fructose-containing medium with cAMP,

Table 2. Growth rate ($\mu\text{m h}^{-1}$) of *A. fumigatus* and *A. niger* when grown on glucose or glycerol minimal medium with cAMP and IBMX alone and in combination

Medium	Growth rate	
	<i>A. fumigatus</i>	<i>A. niger</i>
ASP MM + glucose		
Alone	744 $\pm$ 12	702 $\pm$ 6
+ cAMP*	725 $\pm$ 5	453 $\pm$ 10†
+ 0.5 mM IBMX	679 $\pm$ 13†	628 $\pm$ 12†
+ cAMP* and IBMX‡	654 $\pm$ 2†	286 $\pm$ 14§
ASP MM + fructose		
Alone	692 $\pm$ 2	468 $\pm$ 5
+ cAMP*	682 $\pm$ 14	448 $\pm$ 8†
+ 0.5 mM IBMX	676 $\pm$ 5†	436 $\pm$ 6†
+ cAMP* and IBMX‡	639 $\pm$ 8†§	410 $\pm$ 11†§
ASP MM + glycerol		
Alone	722 $\pm$ 6	533 $\pm$ 1
+ cAMP*	656 $\pm$ 12†	511 $\pm$ 8
+ 0.5 mM IBMX	706 $\pm$ 4	479 $\pm$ 10†
+ cAMP* and IBMX‡	600 $\pm$ 2†§	504 $\pm$ 25†

* cAMP concentration for *A. fumigatus*, 10 mM; for *A. niger*, 1 mM on glucose and 10 mM on fructose and glycerol.

† $P < 0.05$ compared to the growth control (alone).

‡ IBMX concentration, 0.5 mM.

§ Combination is significantly different from both cAMP and IBMX alone, $P < 0.05$.

Table 3. PKA activity, following cAMP addition, in cell extracts from *A. fumigatus* and *A. niger* grown on glucose and glycerol

Time (min)*	Specific activity [U (mg protein) ⁻¹]			
	<i>A. fumigatus</i>		<i>A. niger</i>	
	Glucose	Glycerol	Glucose	Glycerol
0	31.7 $\pm$ 0.9	6.2 $\pm$ 1.3	4.2 $\pm$ 1.5	10.1 $\pm$ 2.1
5	26.0 $\pm$ 1.8	25.8 $\pm$ 2.0†	19.6 $\pm$ 0.3†	13.3 $\pm$ 3.1
15	31.5 $\pm$ 2.8	9.3 $\pm$ 1.6	18.0 $\pm$ 0.6†	7.5 $\pm$ 3.6
30	30.2 $\pm$ 0.8	5.1 $\pm$ 1.6	8.8 $\pm$ 0.6	1.7 $\pm$ 1.0

* Time after addition of 10 mM cAMP to culture.

† Significantly different from 0 time, $P < 0.05$.

only the growth of *A. niger* was significantly inhibited (Table 2). However, on galactose, glycerol and acetate, cAMP did significantly inhibit the growth rate of *A. fumigatus*, whereas the *A. niger* growth rate was inhibited on acetate (Tables 1 and 2). Indeed, on both fructose and glycerol, additive growth rate reduction was seen for *A. fumigatus* grown on a combination of cAMP and IBMX (Table 2). These data together support the idea that carbon catabolite repression may be

involved in the response to exogenous cAMP in *A. fumigatus*.

PKA activity

To explore further the hypothesis that the insensitivity of *A. fumigatus* to the addition of exogenous cAMP may be due to the glucose activation of the cAMP/PKA pathway, kemptide phosphorylation was used to mea-

sure PKA activity in cell extracts of *A. fumigatus* and *A. niger*. It had been previously shown that strains of *A. niger* in which the catalytic subunit of PKA is over-expressed, as a consequence of introduction of additional copies of the gene, exhibit increased kinase activity, as determined by kemptide phosphorylation (Bencina *et al.*, 1997). *A. niger* was grown in minimal medium containing glucose and cAMP was added at time zero prior to cell harvest. Cell extracts from these cultures exhibited a 3.5-fold increase in kinase activity over the baseline at 5 and 15 min following cAMP addition, suggesting that PKA activity is increased by the addition of exogenous cAMP (Table 3). Under the same growth conditions, there was no change in the PKA activity of cell extracts from *A. fumigatus*, although the basal level of kinase activity for *A. fumigatus* was approximately sevenfold higher than that of *A. niger* (Table 3). The high basal level of activity of *A. fumigatus* may be near the organism's maximum, preventing further increases in activity stimulated by the addition of exogenous cAMP. This would be reflected in the failure to see a growth rate reduction in *A. fumigatus* on glucose medium with cAMP.

When the carbon source in the growth medium was changed to glycerol the kinase activity of *A. fumigatus* did respond to treatment with cAMP, showing a fourfold increase (Table 3). Interestingly, the kinase activity in *A. niger* extracts from cells grown on glycerol did not respond to the cAMP pulse. The difference in basal kemptide phosphorylation activity in extracts from *A. fumigatus* grown in glucose versus the basal activity in extracts from cells grown in glycerol supports the hypothesis that PKA activity may be maximal in medium containing glucose and, therefore, refractory to cAMP addition.

Both *A. niger* and *A. fumigatus* exhibited increases in kemptide phosphorylation activity under conditions in which they displayed reductions of growth. These data are consistent with the hypothesis that the cAMP-induced growth reduction is modulated through changes in carbon-source-regulated PKA activity. Although the precise mechanism underlying the carbon-source-dependent effect of cAMP remains to be elucidated, it raises interesting questions regarding the disparate regulation between these two *Aspergillus* species. The pathways and receptors used to sense glucose and other carbon sources may differ between the two species, which may be reflected in their different industrial roles. Although *A. fumigatus* is an important member of the compost cycle, it has little use in industrial processes whereas *A. niger*, which is cosmopolitan in distribution, is an important industrial source for citric acid and enzymes, such as amylase (Raper & Fennell, 1965).

A. fumigatus responds to the addition of extracellular cAMP only when growing on a carbon source that is non-repressing. Our data supports the interpretation that during growth on glucose, the PKA activity is already near maximal, rendering the organism refractory to addition of exogenous cAMP. However, when basal levels of PKA are low, the organism responds to a

pulse of cAMP, and PKA activity transiently increases. The results also suggest that *A. fumigatus* undergoes carbon catabolite repression in a manner similar to that of other fungi, and that glucose itself or intracellular acidification secondary to the metabolism of glucose results in activation of the cAMP/PKA pathway. The interrelationship between cAMP/PKA signalling and pathogenesis of other fungi and the recent finding that components of the pathway are up-regulated in a model of invasive aspergillosis, when coupled with the data presented here, suggest that PKA signalling is a fruitful area for continued study in *A. fumigatus*.

ACKNOWLEDGEMENTS

We would like to thank Jay Card for his photographic assistance. This work was supported in part by National Institutes of Health award AI041119 to J.C.R. and CA61909 to D.S.A.

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Received 18 March 2002; accepted 10 April 2002.