Exogenous cAMP and cGMP modulate branching in *Fusarium graminearum*

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A study was made of the effects of adenosine 3',5'-cyclic monophosphate (cAMP), guanosine 3',5'-cyclic monophosphate (cGMP) and choline on the morphology and growth of a wild-type strain (A 3/5) and a highly branched, 'colonial' mutant strain (C106) of *Fusarium graminearum*. Addition of up to 50 mM-cAMP or cGMP to the medium had no effect on the specific growth rate of strain A 3/5. For strain A 3/5, but not for strain C106, exogenous cAMP caused significant decreases in both mean hyphal extension rate (*E*) and hyphal growth unit length (*G*), i.e. cAMP caused mycelia of strain A 3/5 to branch profusely. By contrast, for both strains, cGMP caused significant increases in both *E* and *G*, i.e. exogenous cGMP caused mycelia to branch more sparsely. The effects of exogenous cGMP and choline in increasing *E* and *G* were synergistic, but the effects of cGMP and choline counteracted the effect of cAMP. The mutant phenotype of strain C106 was not correlated with altered levels of endogenous cAMP or cGMP.

Introduction

Adenine and guanine nucleotides are involved in the regulation of various processes in eukaryotic cells. In particular, adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) often act as second messengers. Adenylate cyclase and phosphodiesterase activities, and cAMP, have been detected in a range of fungi (Flawià & Torres, 1972; Scott & Solomon, 1973; Shaw & Harding, 1983; Ortega-Perez *et al.*, 1983; Rosenberg & Pall, 1983; Reig *et al.*, 1984). In these organisms, cAMP has been implicated in the utilization of exogenous and endogenous carbon sources, conidiation, dimorphism, phototropism (Pall, 1981), regulation of hierarchical hyphal growth (Pall & Robertson, 1986), and the control of hyphal branching (Terenzi *et al.*, 1976) and spore germination (Egidy *et al.*, 1990; Tomes & Moreno, 1990). However, although cGMP is present in fungi (Rosenberg & Pall, 1978, 1979; Eckstein, 1988), its function is unknown. In bacteria, cGMP takes part in processes of repression/derepression and, in some cases, it acts in a direction opposite to that of cAMP (Bernlohr *et al.*, 1974; Gonzalez & Peterkofsky, 1975). In other eukaryotes, cGMP acts as a second messenger, regulating specific enzymes via cGMP specific protein kinase (Newell *et al.*, 1987; Morgan, 1989).

In filamentous fungi, hyphal extension and branch frequency are interdependent (Steele & Trinci, 1975), as shown below:

$$E = G\mu \quad (1)$$

where *E* = mean rate of hyphal extension, *G* = hyphal growth unit length (mean hyphal length per hyphal tip in a mycelium) and μ = specific growth rate. We are interested in the regulation of hyphal extension and branching as well as the way in which these two processes interact and, in this paper, we describe the effects of exogenous cAMP and cGMP on *Fusarium graminearum* and discuss the possible role of these nucleotides as internal regulators of branching in fungi. The studies were made on a sparsely branched, wild-type strain (A 3/5) of *F. graminearum* and on a highly branched, 'colonial' mutant strain (C106) which arose spontaneously from strain A 3/5 during prolonged cultivation in continuous culture; although strains A 3/5 and C106 have the same specific growth rate, C106 forms colonies which expand in radius much more slowly than colonies of A 3/5 (Wiebe *et al.*, 1989). In addition, the interaction between cyclic nucleotides (cAMP and cGMP) and choline, a compound previously shown to cause *F. graminearum* A 3/5 to branch more sparsely (Wiebe *et al.*, 1989), was studied.

Methods

Organism, medium and cultural conditions. *Fusarium graminearum* strains A 3/5 and C106 (a highly branched, 'colonial' mutant strain of A 3/5) were obtained from Mr T. W. Naylor (Marlow Foods, Billingham, UK) and maintained on soil at 4 °C. Soil cultures were prepared by inoculating sterile soil (autoclaved at 121 °C for 1 h on three consecutive days) in bijou bottles with 0.5 ml of a conidial suspension (1×10^6 conidia ml⁻¹) and incubating for 3 d at 25 °C before storing at 4 °C. Spores were harvested (in distilled water) from cultures grown at 25 °C for 7 d on plates of modified Vogel's medium (Vogel, 1956), prepared as described previously (Robson *et al.*, 1987). Macroconidia were filtered through two layers of lens tissue (Whatman), centrifuged at 1500 g for 3 min and washed twice in 10 ml volumes of distilled water. Spore concentration was determined using a haemocytometer and the suspension was diluted in distilled water to give a final concentration of 2×10^3 conidia ml⁻¹. All nucleotides were obtained as their sodium salts (Sigma) with the exception of cAMP (Aldrich). All other chemicals were of analytical grade and, unless otherwise stated, were obtained from BDH. Adenine and guanine nucleotides were prepared as filter-sterilized (Whatman, 0.22 µm) stock solutions (200 mM) in water and stored in aliquots at -20 °C until required. One millilitre of medium containing the nucleotide was prepared by adding an appropriate volume of nucleotide stock solution and 0.02 ml filter-sterilized (Whatman, 0.22 µm) Vogel's salts solution ($\times 50$ final concentration) to 0.5 ml of 3% (w/v) agar (Taiyo powdered agar, Davis Gelatine) containing 2% (w/v) glucose (autoclaved at 115 °C for 10 min). The nucleotide-containing medium was made up to 1 ml with distilled water and was then poured into a 2 × 2 cm compartment in a sterile Sterilin plastic plate (plates divided into 25 such compartments). During preparation, all medium ingredients were kept at 55 °C. Media in the compartments were overlaid with 2 × 2 cm squares of sterile Cellophane (BT 14, British Cellophane) which had been boiled twice for 20 min in distilled water to remove plasticizers. Each Cellophane square was then inoculated with 10 µl of the above spore suspension to give about 20 spores per Cellophane square and the cultures were incubated at 25 °C overnight.

Measurements of growth and morphology of mycelia. Measurements of mycelial morphology were made using a Measuremouse system (Analytical Measuring Systems) and a Nikon microscope linked to a video camera and an Amstrad 1512 PC; measurements of total hyphal lengths were made at a final magnification of $\times 136$ or $\times 336$. Growth was monitored by measuring the total hyphal length of the germlings at 1 h intervals and was followed for at least 5 h. Specific growth rates were calculated from the slopes of $\ln(\text{total germling length})$ vs time, and mean hyphal extension rates were determined as described by Trinci (1974). Hyphal growth unit lengths were determined on germlings which had at least five branches (Trinci, 1973).

Extraction and measurement of cyclic nucleotides. Liquid cultures of *F. graminearum* strains A 3/5 and C106 were grown at 25 °C in 20 ml Vogel's modified medium in 250 ml Nephlos (Trinci, 1972) shake flask cultures at 200 r.p.m. on a rotary shaker with a 2.5 cm stroke. Growth was monitored by measuring optical density with an EEL colorimeter (green filter, about 540 nm) and biomass was harvested when the optical density was between 1.0 and 2.0 (cultures in early exponential phase). Strain A 3/5 was also grown on solid medium for nucleotide determinations; plates of Vogel's modified medium were overlaid with Cellophane, inoculated with 0.2 ml volumes of a 1×10^6 conidia ml⁻¹ suspension spread evenly over the surface of the medium. Plates were incubated at 25 °C and biomass was removed from the Cellophane about 20 h (exponential phase) after inoculation.

For both strains, biomass was collected onto muslin and rapidly washed before immersion in 2.5 ml ice-cold 5% (w/v) trichloroacetic

acid (TCA) and stored at 4 °C for 2 h. The interval between harvesting biomass and immersing it in TCA was typically <15 s. The TCA extract was separated from the biomass by centrifugation (14000 g, 3 min) and the TCA removed from the sample by extracting four times with 5 vols water-saturated diethyl ether. The extract was stored at -20 °C for up to two weeks before assaying for cAMP and cGMP. The pelleted biomass was rinsed and collected on pre-weighed filter papers and dried to constant weight for dry weight determinations. cAMP and cGMP were measured using a competitive binding assay kit obtained from Amersham. Determinations of cAMP and cGMP were performed in duplicate for each sample.

Results

Effects of exogenous cAMP on the morphology and growth of strains A 3/5 and C106

Concentrations of cAMP up to 50 mM had no significant ($P > 0.05$) effect on the specific growth rate of *F. graminearum* strain A 3/5, but caused significant ($P < 0.05$) reductions in mean hyphal extension rate and hyphal growth unit length (Table 1; Fig. 1a). Thus, mycelia of strain A 3/5 treated with cAMP were much more highly branched than untreated mycelia (Fig. 3a). Addition of AMP to the medium also caused a significant ($P < 0.05$) reduction in hyphal growth unit length, but at each concentration tested, this decrease was less than that observed for cAMP (Fig. 1a). By contrast, 2',3'-cAMP had no significant ($P > 0.05$) effect on hyphal growth unit length (Fig. 1a).

The response of strain C106 to cAMP and AMP differed to that observed for strain A 3/5; both nucleotides caused a slight but significant ($P < 0.05$) increase in hyphal growth unit length (Fig. 1b). Thus, unlike the wild-type, cAMP-treated mycelia of C106 were more sparsely branched than control mycelia (Fig. 3b). As with strain A 3/5, 2',3'-cAMP had no significant ($P > 0.05$) effect on the length of the hyphal growth unit of strain C106 (Fig. 1b).

Effects of exogenous cGMP on the morphology and growth of strains A 3/5 and C106

Concentrations of cGMP up to 50 mM had no significant ($P > 0.05$) effect on the specific growth rate of *F. graminearum* A 3/5 (Table 1). However, in contrast to the results obtained with cAMP, cGMP caused significant ($P < 0.05$) increases in the mean hyphal extension rates and hyphal growth unit lengths of both strains (Table 1; Fig. 2a, b). Thus, cGMP caused mycelia of both strains to branch more sparsely than control mycelia (Fig. 3a, b). For both strains, 2',3'-cGMP had no significant ($P > 0.05$) effect on branching but GMP caused a small but statistically significant ($P < 0.05$) increase in hyphal growth unit length (Fig. 2a, b).

Fig. 1. Effect of various concentrations of 3',5'-cAMP (○), 2',3'-cAMP (△) and AMP (□) on the hyphal growth unit length of (a) strain A 3/5 and (b) strain C106. Each result represents the mean of at least 30 germlings from two independent experiments $\pm$ SE.

Fig. 2. Effect of various concentrations of 3',5'-cGMP (○), 2',3'-cGMP (△) and GMP (□) on the hyphal growth unit length of (a) strain A 3/5 and (b) strain C106. Each result represents the mean of at least 30 germlings from two independent experiments $\pm$ SE.

Table 1. Effects of cAMP and cGMP on the specific growth rate, mean hyphal extension rate and hyphal growth unit length of *F. graminearum* A 3/5 cultured at 25 °C on modified Vogel's agar medium

The results are the means of 6 (*) and 20 (†) replicates, respectively, and are shown $\pm$ SE. Figures in the same column with different superscript letters are significantly different ($P > 0.05$).

Treatment	Specific growth rate* (μ , h ⁻¹)	Mean hyphal extension rate* (E , μ m h ⁻¹)	Hyphal growth unit length† (G , μ m)
Control	0.22 $\pm$ 0.01 ^a	67 $\pm$ 3 ^a	323 $\pm$ 13 ^a
10 mM-cAMP	0.25 $\pm$ 0.02 ^a	49 $\pm$ 3 ^b	192 $\pm$ 10 ^b
50 mM-cAMP	0.26 $\pm$ 0.02 ^a	28 $\pm$ 4 ^c	108 $\pm$ 22 ^c
10 mM-cGMP	0.23 $\pm$ 0.01 ^a	86 $\pm$ 8 ^d	382 $\pm$ 25 ^d
50 mM-cGMP	0.24 $\pm$ 0.02 ^a	110 $\pm$ 17 ^e	442 $\pm$ 19 ^e

Synergism between the effects of choline and cGMP on mycelial morphology

Low concentrations (1–5 μ M) of choline, like high concentrations (10–50 mM) of cGMP, cause mycelia of

strain A 3/5 to branch more sparsely than untreated mycelia (Wiebe *et al.*, 1989). To test the hypothesis that choline and cGMP affect branching by influencing a common mechanism, mycelia of strain A 3/5 were treated with combinations of sub-optimal concentrations of choline (0.5 and 1.0 μ M) and cGMP (5 and 10 mM). Such combined treatments resulted in the formation of sparsely branched mycelia with hyphal growth unit values that were significantly ($P < 0.05$) longer than the values which would be predicted if the effects of choline and cGMP were additive (Table 2). Thus, the compounds were synergistic when applied at sub-optimal concentrations. Synergy between the two compounds was most pronounced when the lowest concentrations of choline and cGMP were used, and no synergy was observed when the additives were present in supra-optimal (20 μ M-choline and 25 mM-cGMP) concentrations (Table 2). In the above studies with cAMP and cGMP, the nucleotides tested had no significant ($P > 0.05$) effect on hyphal diameter (results not shown).

Table 2. *Effects of choline and cGMP individually and in combination on the hyphal growth unit length of mycelia of F. graminearum A 3/5 cultured at 25 °C on modified Vogel's agar medium*

Hyphal growth units are the means of at least 11 replicates and are shown $\pm$ SE. Figures in the same column with different superscript letter are significantly different ($P > 0.05$).

Concn of choline (μ M)	Concn of cGMP (mM)	Hyphal growth unit length (G, μ m)	Hyphal growth unit length predicted* from the response of mycelia treated with choline or cGMP (G, μ m)
0	0	305 $\pm$ 13 ^a	—
0.5	0	319 $\pm$ 18 ^b	—
1.0	0	376 $\pm$ 17 ^c	—
20.0	0	426 $\pm$ 21 ^d	—
0	5	342 $\pm$ 14 ^e	—
0	10	377 $\pm$ 15 ^c	—
0	25	422 $\pm$ 23 ^d	—
0.5	5	503 $\pm$ 29 ^f	358 $\pm$ 50 ^g
0.5	10	527 $\pm$ 25 ^g	423 $\pm$ 59 ^d
1.0	5	482 $\pm$ 27 ^f	422 $\pm$ 54 ^d
1.0	10	498 $\pm$ 28 ^f	465 $\pm$ 59 ^h
20.0	25	495 $\pm$ 21 ^f	589 $\pm$ 86 ⁱ

* The predicted effects of cGMP and choline in combination were calculated from the effect of each compound on its own. For example 5 mM-cGMP causes an increase over the control of $342/305 = 1.12$ and therefore, when added to cultures grown on 0.5 μ M-choline, a G value of $319 \times 1.12 = 358 \mu$ m would be predicted.

Table 3. *Effects of cAMP, cGMP and choline individually and in combination on the hyphal growth unit length of mycelia of F. graminearum A 3/5 cultured at 25 °C on modified Vogel's agar medium*

Hyphal growth unit measurements are the means of at least 13 replicates and are shown $\pm$ SE. Figures in the same column with different superscript letter are significantly different ($P > 0.05$). Results are from two experiments.

Concn of choline (μ M)	Concn of cAMP (mM)	Concn of cGMP (mM)	Hyphal growth unit length (G, μ m)	Hyphal growth unit length predicted* from the response of mycelia treated with choline, cAMP or cGMP alone (G, μ m)
0	0	—	347 $\pm$ 14 ^a	—
20	0	—	428 $\pm$ 42 ^b	—
0	10	—	209 $\pm$ 23 ^c	—
0	25	—	96 $\pm$ 8 ^d	—
20	10	—	240 $\pm$ 11 ^{ey}	258 $\pm$ 64 ^g
20	25	—	116 $\pm$ 7 ^f	118 $\pm$ 26 ^f
—	0	0	316 $\pm$ 16 ^h	—
—	0	25	428 $\pm$ 21 ^b	—
—	10	0	161 $\pm$ 10 ^e	—
—	25	0	93 $\pm$ 6 ^d	—
—	10	25	231 $\pm$ 15 ^{ay}	218 $\pm$ 35 ^{aj}
—	25	25	131 $\pm$ 11 ^k	126 $\pm$ 22 ^k

* The predicted effects of the additives in combination were calculated from the effect of each additive on its own (see Table 2).

Interaction between choline and cAMP, and between cAMP and cGMP

cGMP (25 mM) and 20 μ M-choline both partially reversed the effect of cAMP in reducing hyphal growth

unit length of strain A 3/5 and their effect on cAMP-reduced hyphal growth unit length was additive (Table 3), suggesting that there was no interaction between these compounds in counteracting the effect of cAMP.

Fig. 3. Effect of 50 mM-3',5'-cAMP and 3',5'-cGMP on the morphology of (a) strain A 3/5 and (b) strain C106.

Levels of cAMP and cGMP in *F. graminearum* strains A 3/5 and C106

In view of the effects of exogenous cAMP and cGMP on the morphology of mycelia of *F. graminearum* A 3/5 (Figs. 1–3), it was thought that the mutant phenotype of *F. graminearum* C106 might be correlated with increased levels of endogenous cAMP or decreased levels of endogenous cGMP.

However, no significant ($P > 0.05$) difference was observed between the levels of endogenous cAMP in liquid-grown cultures of strain A 3/5 [4.4 ± 0.3 nmol (g dry wt) $^{-1}$; mean $\pm$ SE] and strain C106 [4.5 ± 0.5 nmol (g dry wt) $^{-1}$]; the cAMP level of strain A 3/5 grown in plate cultures [5.9 ± 0.07 nmol (g dry wt) $^{-1}$] was similar to that observed for liquid-grown cultures. Further, endogenous cGMP levels in liquid grown cultures of strain A 3/5 [0.12 ± 0.06 nmol (g dry wt) $^{-1}$; mean $\pm$ SE] and strain C106 [0.11 ± 0.03 nmol (g dry wt) $^{-1}$] were also not significantly different ($P > 0.05$). Thus, the mutant phenotype of strain C106, which is observed in both solid and liquid culture, was not correlated with altered levels of endogenous cAMP or cGMP. Although the level of

endogenous cGMP in liquid-grown cultures of strain A 3/5 was much lower than the level of endogenous cAMP in cultures grown on liquid or solid media, cultures of this strain grown on solid medium contained much higher levels of endogenous cGMP [3.2 ± 1.0 nmol (g dry wt) $^{-1}$].

Discussion

In *Saccharomyces cerevisiae*, cAMP is required for regulation of the cell cycle, and growth is prevented in mutants which contain low levels of cAMP (Matsumoto *et al.*, 1983, 1986). In some filamentous fungi, low levels of cAMP are correlated with increased branching and decreased colony radial growth rate, suggesting that basal levels of cAMP are necessary for normal mycelial development (Terenzi *et al.*, 1974; Scott & Solomon, 1975; Flawià *et al.*, 1977; Rosenberg & Pall, 1979).

In this study it appears that, in the absence of any effect on specific growth rate, increased levels of cAMP elicit changes in mycelial development (Fig. 3a) by causing a decrease in mean hyphal extension rate and an increase in branching. Such a response to exogenous cAMP has previously been reported for *Neurospora crassa*, although, in that study, it was not clear whether specific growth rate was also altered (Mishra, 1976). cGMP affected mycelial morphology of strain A 3/5 (Fig. 3b), in a manner directly opposite to that of cAMP, with both mean hyphal extension rate and branching being increased.

The synergism observed between cGMP and choline (Table 2) strongly suggests that these compounds may affect a common pathway. Choline appears to act primarily by inhibiting branch initiation rather than by stimulating hyphal extension (M. G. Wiebe, G. D. Robson & A. P. J. Trinci, unpublished observations) and it seems likely therefore that cGMP may act in the same manner.

Although very high concentrations of exogenous nucleotides were required to elicit changes in mycelial branching, this is very likely to reflect the relative impermeability of the membrane to such compounds. Similar concentrations of cAMP were required to restore the wild-type phenotype to a mutant (*cr*) of *N. crassa* which contained a reduced level of cAMP (Terenzi *et al.*, 1974; Rosenberg & Pall, 1979) and *S. cerevisiae* *cyr* (Matsumoto *et al.*, 1982). In this regard, it is noteworthy that the analogues 2',3'-cAMP and 2',3'-cGMP had no significant effect on the morphology of mycelia of *F. graminearum* even when present at high concentrations (Figs 1 and 2).

Endogenous cAMP and cGMP levels in the highly branched strain, C106, were found to be the same as for

the wild-type in liquid culture. Hence, unlike the *cr* mutant of *N. crassa*, increased branching was not correlated with altered nucleotide levels. However, although the response of strain C106 to cGMP was similar to that of the wild-type (Fig. 2a, b), its apparent insensitivity to exogenous cAMP (Fig. 1a, b) suggests that the mutation might have affected a cAMP target protein, possibly cAMP-dependent protein kinase, so that the organism responds as if cAMP levels were permanently raised.

In filamentous fungi, hyphal extension and branching are interdependent (equation 1) and the developing mycelium can vary between a slowly extending, highly branched form and a more rapidly extending, more sparsely branched form (Trinci, 1974). This interdependency indicates the existence of an internal regulatory mechanism whereby the rate of hyphal extension can influence the degree of branching (and vice versa). In eukaryotic cells, cAMP acts as a messenger regulating many aspects of cell growth and development by phosphorylating target proteins. Both cAMP-dependent protein kinases and cAMP-dependent phosphorylation of specific proteins have been reported in fungi (Powers & Pall, 1980; Swamy *et al.*, 1985; Behrens & Mazon, 1988; Cherry *et al.*, 1989; Kato *et al.*, 1989; St Leger *et al.*, 1990). cGMP acts as a regulatory molecule and second messenger in eukaryotes and also mediates its effects by protein phosphorylation, although its role generally is poorly understood. In fungi, cGMP has been detected in only a few organisms and has not previously been implicated in any particular function.

One way in which the level of cyclic nucleotides may influence morphology would be by activating or deactivating (by specific phosphorylation) enzymes involved in tip wall growth or branch initiation. It is significant that directly altering the activity of chitin synthase in *F. graminearum* by the non-competitive inhibitor edifenphos (Hinosan), causes a decrease in the mean hyphal extension rate and an increase in branching (without affecting μ), illustrating the potential role of wall biosynthetic enzymes in determining the branch pattern of a fungus (Binks *et al.*, 1991). However, the precise manner in which cyclic nucleotides regulate hyphal extension and branching in fungi is yet to be determined.

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