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Properties of Two Cyclic Nucleotide-Deficient Mutants of *Neurospora crassa*

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Studies on the *crisp-1* (*cr-1*), cyclic adenosine 3',5'-monophosphate (cAMP)-deficient mutants of *Neurospora crassa* were undertaken to characterize the response of these mutants to exogenous cyclic nucleotides and cyclic nucleotide analogs. A growth tube bioassay and a radioimmune assay for cyclic nucleotides yielded the following results. (i) 8-Bromo cAMP and N^6-monobutyryl cAMP but not dibutyryl cAMP are efficient cAMP analogs in *Neurospora*, stimulating mycelial elongation of the *cr-1* mutants. Exogenous cyclic guanosine 3'5'-monophosphate (cGMP) also stimulates such mycelial elongation. (ii) Both cAMP levels and cGMP levels found in *cr-1* mycelia are lower than those in wild type. However, the levels of both cyclic nucleotides are normal in conidia of *cr-1*. The data on *cr-1* mycelia and those reported earlier in *Escherichia coli* (M. Shibuya, Y. Takebe, and Y. Kaziro (Cell **12**:521-528, 1977) show a previously unexpected relationship between cAMP and cGMP metabolism in microorganisms. The semi-colonial morphology of another adenylate cyclase-deficient mutant of *Neurospora*, *frost*, was not corrected by exogenous cyclic nucleotides or by phosphodiesterase inhibitors indicating that the *frost* morphology is probably not caused by low endogenous cAMP levels. The low adenylate cyclase activity and the abnormal morphology of *frost* may be related separately to the linolenate deficiency reported in the mutant.

Investigations of the role of cyclic 3',5'-AMP (cAMP) and cyclic 3',5'-GMP (cGMP) in fungi have involved several organisms including *Neurospora crassa* (7, 8, 11, 13-16, 19-21). Two mutants have been isolated in *Neurospora* which appear to be defective in cAMP metabolism. The *crisp-1* (*cr-1*) mutants, which have a colonial morphology, have been reported by Terenzi et al. (19, 20) to have reduced adenylate cyclase activity and low endogenous levels of cAMP. Terenzi et al. (19, 20) also reported that exogenous cAMP or N^6,$O^{2'}$-dibutyryl cAMP at least partially correct the aberrant morphology of *cr-1*.

The other mutant of *Neurospora*, *frost*, (*fr*), has also been reported to have reduced mycelial adenylate cyclase activity (13) and low endogenous cAMP levels (14). The *fr* mutant is deficient in linolenic acid and has a semi-colonial morphology which is reported to be corrected by either exogenous linolenic acid or theophylline (13), an inhibitor of cAMP phosphodiesterase (15), but not by exogenous cAMP (16). Scott and Solomon (16) suggest that the reason *fr* does not respond to exogenous cAMP is because *Neurospora* is impermeable to the cyclic nucleotide.

The experiments described in this paper were initiated to examine the problem of the permeability of *Neurospora* to exogenous cAMP. A growth tube bioassay was used to quantitate the response of *cr-1* and *fr* to exogenous cyclic nucleotides. The results presented below suggest that there is some relationship between the metabolism of cAMP and cGMP in *Neurospora*. Although a relationship of this kind has not before been suggested in fungi, a similar situation has been described in *Escherichia coli* (17).

MATERIALS AND METHODS

The wild-type *Neurospora crassa* strain St. L. 74A was used in this work. The mutant strains *cr-1(B122)*, *cr-1(C-Ex-11-67)*, *cr-1(B74)*, *cr-2(R2445)*, *cr-3(R2509)*, and *fr(B110)* were all obtained from the Fungal Genetics Stock Center. The *cr-1(B123)* strain carried an *al(15300)* marker and was the gift of Alice Schroeder. All of the mutant strains except for the *cr-1(B122)* mutant are of mixed or unknown background (1). The *cr-1(B122)* mutant was isolated from UV light-treated St.L.74A and crossed back into the St.L.74A strain (9).

All nucleotides, theophylline, and caffeine were obtained from the Sigma Chemical Co. Conidia were grown and germinated and cyclic nucleotides were assayed as previously described (8, 11).

Growth tubes used in these experiments were of a general design described by Ryan et al. (12). The large growth tubes were 55 cm long and had an inside diameter of 1.0 cm. The smaller tubes were 23 cm long with an inside diameter of 0.9 cm.

Neurospora was grown in the N medium of Vogel (22), supplemented with 2% sucrose and 2% agar which were all autoclaved together. The cyclic nucleotides and their derivatives were either filter sterilized or boiled for 10 min and added separately to the medium. There was no difference in activity when the nucleotides were boiled or filter sterilized. The large growth tubes each contained 12 ml of medium, and 5 ml was used in each of the small growth tubes.

The growth tube bioassay consisted of inoculating a particular tube at one end with a strain of *Neurospora* and measuring the distance which the mycelial front progressed down the tube as a function of time. All experiments were carried out at 25°C, and measurements were made approximately every 24 h.

RESULTS

The effects of exogenous cAMP on several strains of *Neurospora* as assayed in growth tubes are shown in Table 1. Each value in Table 1 and in all subsequent tables depicting growth tube experiments is an average of at least four determinations. The growth tube data were very reproducible. The largest variation, about ±10% of the wild-type standard, was seen with the *frost* (*fr*) mutant. As previously reported by Terenzi et al. (19), exogenous cAMP was active in stimulating mycelial elongation of *cr-1* mutants but had little or no effect on the *cr-2* or the *cr-3* mutants (Table 1). (The *cr-2* and *cr-3* mutants have similar morphology to that shown by *cr-1*, are linked to the *cr-1* mutation, but are nonallelic.) Neither adenosine nor 5'AMP had any visible effect on the growth of the *cr-1* mutant. Caffeine and theophylline, inhibitors of cAMP phosphodiesterase (15), were individually ineffective in stimulating elongation of *cr-1*, but theophylline in conjunction with cAMP was more active than cAMP alone.

TABLE 1. *Effects of exogenous cAMP and inhibitors of cAMP phosphodiesterase on cr mutants of Neurospora*

Strain	Mycelial elongation rate (% wild-type growth)
St.L.74A (wild type)	100 (2.6 mm/h)
cr-1(B123) al(15300)	5
cr-1(B122), FGSC 804	5
cr-1(C-Ex-11-67), FGSC 814	8
cr-1(B74), FGSC 826	6
cr-1(B123), *al*(15300)	
+ 1 mM cAMP	10
+ 10 mM cAMP	30
+ 30 mM cAMP	38
+ 10 mM adenosine	5
+ 10 mM 5'-AMP	5
cr-2(R2455), FGSC 2208	45
+ 30 mM cAMP	48
cr-3(R2509), FGSC 2329	24
+ 30 mM cAMP	24
cr-1(B123) *al*(15300)	
+ 5 mM caffeine	2
+ 15 mM caffeine	2
+ 50 mM caffeine	2
cr-1(B123) *al*(15300)	
+ 10 mM theophylline	5
+ 20 mM theophylline	5
+ 30 mM theophylline	5
+ 10 mM theophylline + 10 mM cAMP	47

TABLE 2. *Effects of cAMP analogs on cr-1 mutants of Neurospora*

Strain	Mycelial elongation rate (% wild-type growth)
cr-1(B123) *al*(15300)	5
+ 10 mM cAMP	30
+ 10 mM dibutyryl cAMP	20
+ 0.05 mM N^6-monobutyryl cAMP	10
+ 0.1 mM N^6-monobutyryl cAMP	14
+ 1.0 mM N^6-monobutyryl cAMP	58
+ 0.05 mM $O^{2'}$-monobutyryl cAMP	4
+ 0.1 mM $O^{2'}$-monobutyryl cAMP	5
+ 1.0 mM $O^{2'}$-monobutyryl cAMP	6
+ 0.05 mM 2'-deoxy cAMP	3
+ 0.1 mM 2'-deoxy cAMP	11
+ 1.0 mM 2'-deoxy cAMP	3
+ 0.05 mM 8-bromo cAMP	2
+ 0.1 mM 8-bromo cAMP	10
+ 1.0 mM 8-bromo cAMP	53
cr-1(B122), FGSC 804	5
+ 1.0 mM 8-bromo cAMP	73
cr-1(C-Ex-11-67), FGSC 814	8
+ 1.0 mM 8-bromo cAMP	54
cr-1(B74), FGSC 826	6
+ 1.0 mM 8-bromo cAMP	68

The effects of several cAMP analogs in stimulating mycelial elongation of *cr-1* as measured in growth tubes are shown in Table 2. Contrary to what has been reported (19–21), N^6,$O^{2'}$-dibutyryl cAMP was less effective in stimulating growth of *cr-1* than was cAMP; however, one of the monobutyryl derivatives, N^6-monobutyryl cAMP, was extremely active in stimulating mycelial elongation of the *cr-1* mutant. 8-Bromo cAMP, which is another effective cAMP analog in mammalian cells (4, 10), was also very active in stimulating mycelial elongation in the *cr-1* mutants. Even at low concentrations where the mycelial elongation rate was not affected, cAMP, N^6,$O^{2'}$-dibutyryl cAMP, N^6-monobutyryl cAMP, and 8-bromo cAMP were found here to cause the *cr-1* cultures to be more wild type in appearance, showing aerial hyphae and

showing little of the early conidiation characteristic of the *cr-1* phenotype (9). Neither $O^{2'}$-monobutyryl cAMP nor 2'-deoxy cAMP had any visible effect on the morphology of the *cr-1* mutant.

The effects of cGMP and some of its analogs on the growth of the *cr-1* mutant as measured in growth tubes are depicted in Table 3. Surprisingly, at comparable concentrations, cGMP was more active in stimulating *cr-1* mycelial elongation than cAMP. N^2-monobutyryl cGMP and 8-bromo cGMP had only a slight effect on *cr-1* morphology and $N^2,O^{2'}$-dibutyryl cGMP and guanosine had no detectable effect on the mycelial elongation rate of the *cr-1* mutant. Exogenous 8-bromo cAMP and cGMP together were no more effective in correcting *cr-1* morphology than either cyclic nucleotide alone.

TABLE 3. *Effects of cGMP and cGMP analogs on cr mutants of Neurospora*

Strain	Mycelial elongation rate (% wild-type growth)
cr-1(B123) *al*(15300)	5
+ 0.5 mM cGMP	28
+ 1.0 mM cGMP	51
+ 10 mM cGMP	63
+ 1.0 mM dibutyryl cGMP	6
+ 1.0 mM N^2-monobutyryl cGMP	13
+ 0.5 mM 8-bromo cGMP	13
+ 1.0 mM 8-bromo cGMP	13
+ 0.5 mM 8-bromo cAMP + 0.5 mM cGMP	14
cr-2(R2455), FGSC 2208	45
+ 10 mM cGMP	48
cr-3(R2509), FGSC 2329	24
+ 10 mM cGMP	24

The fact that exogenous cGMP corrected the *cr-1* phenotype led us to measure the endogenous cGMP levels in the mutants. Since we reported earlier (11) that mycelial cAMP levels in *cr-1* are reduced but conidial levels are normal, endogenous cGMP was measured over the germination period and in mycelia. As shown in Fig. 1, cGMP levels are as low as cAMP levels in *cr-1*(B123) mycelia and normal in germinating conidia. The levels of both cyclic nucleotides began to drop 12 h after the conidia were suspended in medium, and by 15 h the cyclic nucleotides reached their lowest levels. As with *cr-1*(B123), mycelial cAMP and cGMP levels in three other *cr-1* mutants were low (Table 4). The conidial levels were normal, showing a similar time change of cyclic nucleotide levels to those shown with *cr-1(B123)* (data not shown). In wild type, the endogenous levels of both cyclic nucleotides remained fairly constant over the 20-h period (Fig. 1) (8, 11).

The effects of exogenous cyclic nucleotides on

TABLE 4. *Endogenous cyclic nucleotide levels in wild-type and cr-1 strains of Neurospora*

Strain	Cyclic nucleotide levels[a] [fmol/mg (dry wt)]	
	cAMP	cGMP
74A (wild type)	490	419
cr-1(B123) *al*(15300)	31	48
cr-1(B122), FGSC 804	111	58
cr-1(C-Ex-11-67), FGSC 814	122	63
cr-2(R2455), FGSC 2208	245	
cr-3(R2509), FGSC 2329	227	
fr(B110), FGSC 102	376	

[a] Cyclic nucleotides were measured in 20-h mycelia.

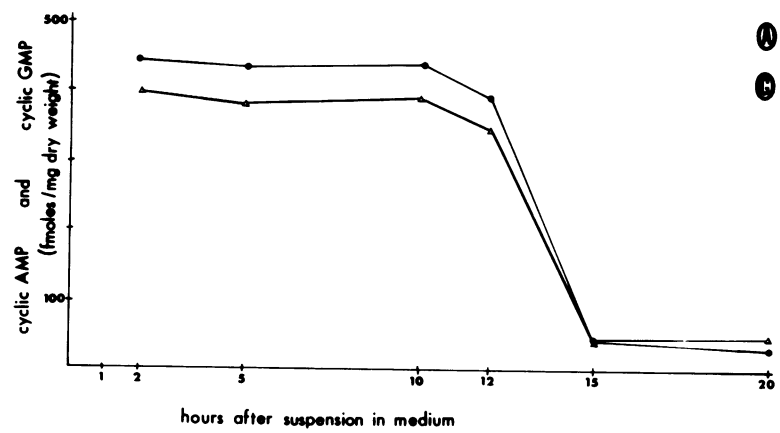

FIG. 1. *Endogenous cAMP (●) and cGMP (▲) levels during the germination of the cr-1(B123) mutant of Neurospora. Endogenous cAMP (A) and cGMP (G) in wild-type mycelia are indicated by open circles. Each point represents the average value of three separate determinations.*

the growth of the *fr* mutant of *Neurospora* are shown in Table 5. The mycelial elongation rates with *frost* were more variable both from race tube to race tube and from experiment to experiment, than were the experiments with other strains. Exogenous cAMP, cGMP, and their analogs did not stimulate mycelial elongation in the *fr* mutant, but, as reported by Scott (13), linolenic acid was effective in correcting its morphology. Theophylline and caffeine, which have been shown to inhibit the cAMP phosphodiesterase of *Neurospora* (15), had no effect on the rate of mycelial elongation of the *fr* mutant (Table 6). Because of the variability seen with the effects of theophylline and caffeine on the *fr* mutant, statistical analysis of the data is shown. Theophylline and cAMP together were also ineffective in stimulating mycelial elongation in the *fr* mutant. Exogenous cGMP, at high concentration, actually inhibited the growth of the *fr* mutant. The inhibition of growth by cGMP was not totally unexpected as Mishra (7) has reported that high concentrations of either cAMP or cGMP inhibit the growth of wild-type *Neurospora crassa* (Table 5).

The finding that cGMP inhibited the growth of *frost* suggested that, if the growth of the mutant could be completely inhibited by cGMP or one of its analogs, then mutants resistant to exogenous cGMP might be selected. Such mutants might be defective in a cGMP receptor protein. Work of this nature has been done in mammalian cells by Coffino and co-workers where some of the cells resistant to the effects of exogenous cAMP were found to be cAMP receptor mutants (2). Unfortunately, as depicted in Table 5, none of the cGMP analogs tested was effective enough in inhibiting the growth of the *fr* mutant to provide a good selection technique.

TABLE 5. *Effects of cyclic nucleotides on the fr and wild-type strains of Neurospora*

Strain	Mycelial elongation rate (% wild-type growth)
frost(B110), FGSC 102	27
+ 0.1 mM linolenic acid	44
+ 1 mM cAMP	28
+ 10 mM cAMP	29
+ 30 mM cAMP	23
+ 10 mM cGMP	20
+ 30 mM cGMP	5
+ 10 mM guanosine	25
+ 1.0 mM N^2-monobutyryl cGMP	26
+ 1.0 mM $O^{2'}$-monobutyryl cGMP	26
+ 1.0 mM 8-bromo cGMP	24
St.L.74A (wild type)	100
+ 10 mM cGMP	76
+ 10 mM cAMP	67

TABLE 6. *Effects of theophylline and caffeine on the fr mutant of Neurospora*

Strain	Mycelial elongation rate[a] (% wild-type growth)
frost	42 ± 5.4
+ 5 mM caffeine	33 ± 3.3
+ 15 mM caffeine	35 ± 2.3
+ 50 mM caffeine	36 ± 7.7
+ 10 mM theophylline	42 ± 11.8
+ 20 mM theophylline	40 ± 8.7
+ 30 mM theophylline	43 ± 6.9
+ 10 mM theophylline + 10 mM cAMP	37 ± 0.5

[a] The values listed represent the mean ± standard deviation of four to nine separate determinations.

DISCUSSION

The response as measured in growth tubes of *Neurospora* to exogenous cAMP and its analogs is strikingly similar to reports on the effectiveness of cAMP analogs in mammalian cell culture. Those cAMP analogs which were active in stimulating mycelial elongation of *cr-1* are also reported to be effective cAMP analogs in mammalian cells (3, 4, 10). N^6-monobutyryl cAMP and 8-bromo cAMP were more active in correcting the *cr-1* phenotype than was cAMP at comparable concentrations (Table 2). The effectiveness of these compounds in mammalian systems has been reported to be due to their resistance to phosphodiesterase degradation and their ability to activate cAMP-dependent protein kinase (4, 6, 10). Similar properties may explain why these compounds are effective cAMP analogs in *Neurospora*. Those compounds which were not effective in reverting the *cr-1* phenotype, adenosine, 5'-AMP, $O^{2'}$-monobutyryl cAMP, and 2'-deoxy cAMP, are not effective cAMP analogs in mammalian systems (4, 6, 10).

The one analog which is effective in mammalian systems but is relatively ineffective in *Neurospora* is N^6,$O^{2'}$-dibutyryl cAMP. In animal cells the dibutyryl cAMP is hydrolyzed to the N^6-monobutyryl derivative which is the active cAMP analog (5). Presumably *Neurospora* lacks the esterase necessary for this hydrolysis. Care must be taken when using dibutyryl cAMP because in aqueous solution it spontaneously hydrolyzes to the N^6-monobutyryl derivative (18). The greater activity with dibutyryl cAMP than with cAMP in *Neurospora* reported by Terenzi et al. (19, 20) may be due to dibutyryl cAMP which is contaminated with the active N^6-monobutyryl derivative.

The response of the *cr-1* mutants to exogenous cGMP (Table 3) was totally unexpected. The ability of cGMP to stimulate mycelial elongation

of *cr-1* becomes less surprising in view of the low endogenous levels of cGMP found in the mycelia of the *cr-1* mutants (Table 4). The fact that the *cr-1* mutation causes low levels of both cAMP and cGMP suggests several possibilities as to the nature of the *cr-1* lesion. It may be that the adenylate cyclase and the guanylate cyclase have some common component which is defective in *cr-1*. Alternatively, a deficiency of one cyclic nucleotide may result in a deficiency of the other. It is also conceivable that the *cr-1* lesion is a pleiotropic mutation such as a membrane defect which might affect both cyclic nucleotides.

Whatever the nature of the *cr-1* lesion, it seems clear that low cyclic nucleotide levels can lead to aberrant morphology. This does not prove, however, that either cyclic nucleotide has a physiological role in controlling morphology in *Neurospora*.

The *cr-1* mutants of *Neurospora* show some striking similarities to the *cya* mutants of *E. coli*. Both mutants are defective in adenylate cyclase activity (17, 20), and both have low cAMP levels. We show here that *cr-1* mutants also have low cGMP levels, and Shibuya et al. (17) report that the *cya* mutants also have low cGMP levels. The finding that a single gene controls both cAMP and cGMP levels in two phylogenetically distant microorganisms, *Neurospora* and *E. coli*, suggests a previously unsuspected relationship between the synthesis of the two cyclic nucleotides in microorganisms.

The nature of the *fr* mutation is not immediately obvious. The semi-colonial morphology of the *fr* mutant was not corrected by either exogenous cAMP or its analogs or by the phosphodiesterase inhibitors caffeine or theophylline. In light of these data it seems unlikely that the abnormal morphology of *fr* is caused by low endogenous cAMP levels, but the semi-colonial morphology and the low adenylate cyclase activity probably are due separately to an as-yet-undetermined defect related to the linolenate deficiency.

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