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Absence of Significant Light-Induced Changes in cAMP Levels in Sporangiophores of *Phycomyces blakesleeanus*

LESLIE S. LEUTWILER* AND MICHAELA BRANDT

Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125

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We were unable to find transient changes in the amount of cAMP or cGMP that had been proposed to mediate the light-growth response in sporangiophores of *Phycomyces blakesleeanus*.

Sporangiophores (SPHs) of *Phycomyces blakesleeanus* show several responses to blue light, among which are the light-growth response (a transient increase in growth rate) and phototropism. Both responses are sensitive over a wide range of light intensities (10^{-9} to 6 W/m^2) and show action spectra which implicate a flavin photoreceptor (4, 5, 10). It has been reported (2, 3) that within 0.5 min after the onset of illumination, the cAMP content of the SPH decreases by more than 60% and then returns within the next 2 min to its original value. Cohen (2) proposed that this modulation is an important link in the transduction chain between absorption of light and the transient change in growth rate which occurs 4 to 5 min later.

Since the light-growth response of the SPH is well characterized (7–9) and behavioral mutants are available (1), we initiated this study to investigate the biochemical events leading to the light responses. We began by looking for changes in levels of cAMP or cGMP that could be correlated with a step up in light intensity. We were unable to find such changes, even under conditions similar to those reported by Cohen (2, 3). We did find that it was extremely important to standardize both growth and experimental conditions. One modification in the method of Cohen was the synchronization of SPH growth. Otherwise, we could not define any specific differences in methodology that would account for the different results obtained.

The standard wild type of *P. blakesleeanus* used in this work was strain NRRL 1555(−) from the Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill. To obtain a synchronized population of SPHs, the method of Russo (11) was used. Approximately 400 heat-shocked (5 to 10 min at 47°C) spores were spread on each of several petri plates (100 by 15 mm) containing 25 ml of potato dextrose agar and thiamine (0.5%). The plates were placed under 1-liter beakers in a shallow pan of water so that no gas exchange would take place. After 60 h of incubation at 19°C in the dark, the mycelia were illuminated with white light (two 24-W Cool White fluorescent lamps; total intensity, 1 W/m^2). SPHs developed to stage I within the next 36 h. The beakers were then removed, and the SPHs developed to stage IVb within approximately 24 h, at which time they measured between 3.5 and 4.0 cm in length.

Twelve plates each containing about 1,000 synchronized stage IVb SPHs were placed in an illumination chamber and dark adapted for 40 min. Three plates were harvested in the dark, and the light was switched on (t = 0). Three plates were harvested at 0.5, 0.75, and 5 min. The blue light source consisted of 16 40-W tungsten-filament incandescent bulbs as described by Jayaram et al. (6).

The SPHs from three plates (approximately 1 g [total fresh weight]) were harvested by plunging them into liquid nitrogen. The upper 2.4 cm was cut off, ground to a fine powder in a mortar containing liquid nitrogen, and homogenized in 10 ml of ice-cold 0.6 N perchloric acid to which trace amounts of [^{3}H]cAMP (11,100 dpm; 0.3 pmol) and [^{3}H]cGMP (11,100 dpm; 0.6 pmol) had been added (amounts sufficiently small to be undetectable in the assay). The mixture was centrifuged, and the pellet was dried in a 70°C oven for approximately 24 h and then weighed. The supernatant fraction was neutralized to pH 6.0 to 6.2 with 6 M KOH, incubated on ice for 1 h, and centrifuged to remove the $KClO_4$ precipitate. Part of the supernatant fraction (1 ml of crude extract) was frozen for future assay.

The rest of the supernatant fraction was loaded on a Bio-Rad AG 1-X8 column (200 to 400 mesh, formate form, 10 cm). The loaded column was washed with 15 ml of distilled water followed by 30 ml of 0.1 N formic acid (pH 2.3). The cAMP fraction was eluted with 15 ml of 2 N formic acid (pH 1.5). The cGMP fraction was eluted with 35 ml of 4 N formic acid (pH 1.1). Both fractions were lyophilized and redissolved

TABLE 1. cAMP Extracted from four batches of SPHs with or without 500 pmol of authentic cAMP and from the extraction solution containing 500 pmol of authentic cAMP

Extract	cAMP recovery (pmol) for following batch:					% Recovery	
	SPHs		SPHs + cAMP		cAMP extraction solution	Batches 1–4	Extraction solution
	1	2	3	4			
Crude	364	359	692	556	481	52	96
Pure	431	402	939	812	524	92	105

in 1 ml of 0.05 M sodium acetate buffer, pH 6.2. Recovery for cAMP was 80% and for cGMP was 65% as judged from the ^3H content of the fractions.

Duplicate assays for each cyclic nucleotide were performed on both the crude and purified extracts, using the radioimmunoassay method of Steiner et al. (12) with kits supplied by New England Nuclear Corp.

A standard curve was prepared relating true dry weight to the weight of the dried perchloric acid pellet. This modification was used because of the necessity for immediate acid fixation of the samples after harvest. Therefore, cAMP levels are reported as picomoles per gram (dry weight) of the perchloric acid precipitate. This represents approximately 85% of the true dry weight and about 10% of the fresh weight.

To check for cAMP authenticity, purified samples were treated with phosphodiesterase and chromatographed with authentic samples of cAMP and cGMP. To check the accuracy of the procedure, unlabeled internal standards of cAMP were added to SPHs before extraction. Four batches of SPHs (three plates of SPHs per batch) were harvested: batches 1 and 2 were treated in the normal way; batches 3 and 4 were mixed and divided into two equal fractions. A 500-pmol amount of authentic cAMP was added to each batch. A similar amount of cAMP was added to a sample of the extraction solution. The results for both the crude and purified extracts are shown in Table 1.

Assuming that X = amount of cAMP in one batch of SPHs before extraction and α = percent recovery of cAMP, the amount of cAMP present in batch 1 and batch 2 after extraction is:

$$\alpha X = A \quad (1)$$

Similarly, the amount of cAMP in batch 3 and batch 4 is:

$$\alpha(X + 500) = B \quad (2)$$

Substitution of equation 1 into equation 2 gives:

$$\alpha = \frac{B - A}{500} \quad (3)$$

The mean value for the amount of cAMP detected in the crude extracts from batches 1 and 2 was 362 pmol (A) and for batches 3 and 4 was 624 pmol (B). This implies (equation 3) that the amount of cAMP recovered from the crude extract was 52%. A similar calculation for the purified extract implies a 92% recovery. Since recovery when only authentic cAMP was present was 96%, it appears that something in the crude extract is inhibiting the binding of cAMP to the antibody. Therefore, it was necessary to purify the cAMP before assay.

This experiment also gave an indication of the variation inherent in the procedure. cAMP values for identical samples (batches 3 and 4) showed standard deviations of the mean of slightly more than 10% for the crude and 7% for the pure extracts. Because of this variation, it is difficult to draw any conclusions from the results of one experiment, unless differences in cAMP content are large.

We found no significant change in cAMP concentration after a step up of light intensity from the dark to a level above that required to saturate the light-growth response. Table 2 shows the results of nine separate experiments.

It is possible that the regulation of cyclic nucleotide levels takes place only in the growing zone of the SPH (located in the 3-mm area directly below the sporangium). Evidence for this proposal comes from the cytochemical localization of adenylate cyclase only in the growing zone and its absence in the nongrowing areas of the SPH (2, 13). Assuming that 12.5% of the cAMP in a 24-mm SPH is present in the 3-mm

TABLE 2. Relative amounts of cAMP and cGMP after a step up in light intensity from dark to 0.13 W/m^2 at time zero[a]

Time (min)	Relative amt ± SD	
	cAMP	cGMP
0	1.00 ± 0.05	1.00 ± 0.05
0.5	1.17 ± 0.18	1.16 ± 0.02
0.75	1.18 ± 0.11	1.28 ± 0.33
5.0	1.14 ± 0.15	1.08 ± 0.10

[a] Absolute amounts of cAMP and cGMP initially present were 3,000 and 350 pmol/g (dry weight), respectively.

growing zone and that the concentration of cAMP in the growing zone decreases by 60% after illumination, we should observe a 7.5% decrease in the cAMP concentration of a 24-mm SPH. According to the data in Table 2, 0.5 min after illumination there was a 17% increase in the mean cAMP concentration with an 18% standard deviation of the mean. At 0.75 min, there was an 18% increase and an 11% standard deviation of the mean. A 7.5% decrease is 1.4 to 2.3 standard deviations away, and the chance of our being off by this much is small, less than 10%. (It should be noted that the measurements made by Cohen [2, 3], in which a 60% decrease in the cAMP content was reported, were made not with growing zones but with whole SPHs.) There also was an increase in the mean cGMP concentration as shown in Table 2. Again, the differences may not be statistically significant. Our evidence suggests that neither cAMP nor cGMP are involved in light transduction in *P. blakesleeanus*.

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