Cyclic AMP Is Not Involved as a Second Messenger in the Response of Soybean to Infection by *Phytophthora megasperma* f. sp. *glycinea*

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cAMP levels were monitored in soybean (*Glycine max*) infected with race 1 of *Phytophthora megasperma* f. sp. *glycinea* and in soybean cell suspension cultures treated with a glucan elicitor from *P. megasperma*. While cAMP could be readily detected in soybean hypocotyls using a radioimmunoassay, no correlation was observed between infection and cAMP levels. No cAMP was detected in suspension cultured soybean cells. These results suggest that cAMP does not play a significant role in the plant's response to fungal attack, or the response of suspension cultured cells to elicitor.

Introduction

Upon infection of soybean (*Glycine max* [L.] Merr.) hypocotyls with *Phytophthora megasperma* f. sp. *glycinea*, the glyceollin isomers I–III accumulate as major phytoalexins at the infection site [1]. It was shown by pulse and pulse-chase experiments that the level of glyceollin is determined predominantly by its rate of synthesis [1]. This conclusion was further supported by the observation that large increases and subsequent decreases of the enzymes phenylalanine ammonia-lyase and chalcone synthase, which are involved in glyceollin biosynthesis, occur after infection [2]. Since the increase in PAL activity is due to *de novo* synthesis of the enzyme [2], the plant/pathogen interaction obviously triggers a chain of events which ultimately leads to an initiation of protein synthesis. The question therefore arises what molecules are involved in this signal transfer.

Adenosine-3′:5′-cyclic monophosphate (cAMP) has been found to have various functions in animals and microorganisms [3]. In particular, cAMP serves as a second messenger in animal systems [4]. The occurrence of cAMP in numerous plants has also been substantially documented [5]. However, the physiological role of this compound in plants is still unclear.

Two papers have dealt with a possible role cAMP in the response of plants to infection by viruses. Tu [6] reported a decrease in cAMP in clover leaves after infection with clover yellow mosaic virus. However, only one unspecified time point was examined, thus an earlier or later increase in cAMP levels might have been missed. Recently, Rosenberg *et al.* [7] described experiments which suggest that cAMP might play a role in mediating the production of antiviral factors in tobacco leaves infected with tobacco mosaic virus. We were interested in testing the hypothesis that cAMP might serve in an analogous role in the response of soybeans to infection with *P. megasperma* f. sp. *glycinea*.

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Materials and Methods

Fungal culture

Phytophthora megasperma Drechs. f. sp. *glycinea* Kuan and Erwin race 1 was obtained from B. L. Keeling (Stoneville, MS, USA) and was maintained on lima bean agar. Cultures for inoculation of plants were grown as described [9] on asparagine medium [10].

Soybean seedlings

Seeds of soybean (*Glycine max* [L.] Merr., cv. Harosoy 63) were obtained from R. I. Buzell (Harrow, Ontario, Canada). Seedlings were grown in vermiculite as described previously [9].

Soybean cell suspension cultures

Soybean cell suspension cultures initiated from callus cultures of Harosoy 63 were grown as de-

scribed [11] in medium I containing 1 mmol l^{-1} $CaCl_2$ [12].

Chemicals

Lima bean agar was purchased from Difco (Detroit, MI, USA). cAMP, [^{3}H]cAMP, and [^{125}I]-succinyl-cAMP-tyrosine methyl ester were obtained from New England Nuclear (Dreieich, FRG). Antisera to cAMP were obtained either from New England Nuclear, or from K. Rissler (Universität Freiburg, FRG). Beefheart phosphodiesterase (E. C. 3.1.4.17) was from Boehringer Mannheim (Mannheim, FRG) and rabbit gamma globulin from Sigma München (München, FRG). A glucan elicitor isolated from *P. megasperma* was donated by J. Ebel (Universität Freiburg, FRG). Other chemicals used were of at least analytical grade.

Hypocotyl inoculation procedure

Hypocotyls of intact five day old soybean seedlings were inoculated with mycelia of *P. megasperma* f. sp. *glycinea* race 1 by the surface wound method previously published [13]. Ten hypocotyls were used for each treatment. Controls consisting of untreated hypocotyls and wounded hypocotyls inoculated with sterile H_2O were also included in each assay.

Treatment of soybean cell suspension cultures

Phenylalanine ammonia-lyase was induced in soybean cell suspension cultures by treatment with a glucan elicitor from *P. megasperma* as follows. Cell cultures used for this experiment had a conductivity of the medium of about 2 mmho. Cell samples (2 g fr. wt.) were transferred to 40 ml fresh medium in a 200 ml Erlenmeyer flask. The transferred cells were pre-incubated 10 h in the dark at 25 °C. Cultures were then inoculated with 8 mg of elicitor (about 25% glucose equivalents) dissolved in one ml sterile water. Control flasks received one ml of sterile water. After incubation as above for various times, the cells were harvested as described [14] and stored at −70 °C until needed. Phenylalanine ammonia-lyase was assayed as described [14].

Extraction and quantitation of cAMP

Hypocotyl segments approximately 1 cm long centered on the wound area were excised, and the mycelia on the wound removed. The hypocotyl segments were immediately frozen in liquid nitrogen and then pulverized using a Mikro-Dismembrator® (B. Braun, Melsungen, FRG) equipped with a tungsten carbide ball. The frozen tissue powder was immediately suspended in 1 ml of 7% (v/v) $HClO_4$. Once the powder suspension had melted, it was centrifuged at 8800 × *g* for 4 min and the supernatant decanted. The $HClO_4$ in the supernatants was neutralized by addition of solid $KHCO_3$. The resulting precipitate was removed by centrifugation and discarded. The supernatants were stored at −70 °C until analyzed for cAMP. Soybean cells (1 g fr. wt.) from suspension culture experiments were similarly extracted with 1.5 ml of 7% (v/v) $HClO_4$. Recovery of cAMP from the tissue extracts was measured using [^{3}H]-cAMP and found to be about 62% for hypocotyl extracts and 69% for extracts of soybean cells from suspension culture.

A radioimmunoassay was used to quantitate cAMP in the plant tissue extracts. Aliquots (100 µl) of the tissue extracts were assayed directly. The radioimmunoassay was carried out in one of two ways, depending on the antiserum used. With the antiserum obtained from New England Nuclear, the

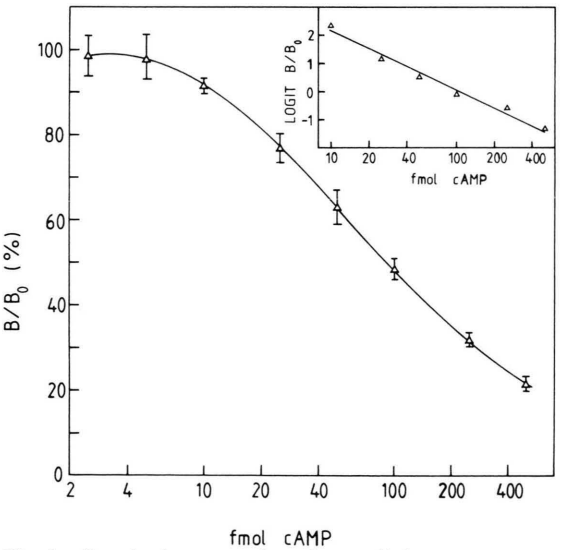

Fig. 1. Standard curve for the radioimmunoassay of cAMP. Each point represents the average, with standard deviations, of seven independent assays with duplicates for each value. B_0 = cpm of tracer bound in the absence of exogenous cAMP, B = cpm of tracer bound in the sample. Logit $B/B_0 = \ln [(B/B_0)/(100\text{-}B/B_0)]$, where B/B_0 is expressed as a percentage.

radioimmunoassay was carried out as described for acetylated samples [15].

This assay was modified as follows when using the antiserum supplied by K. Rissler. The latter antiserum was used at a dilution of 1:60000 in 0.05 M sodium acetate (pH 6.2). After incubation of the tubes containing acetylated sample, tracer and antiserum for 16 to 18 h at 8 °C, 100 µl of rabbit gamma globulin (5 mg/ml) were added to each tube followed immediately by 0.5 ml of a 90% saturated ammonium sulfate solution. The samples were mixed well, allowed to stand for 1 h at 8 °C, and then centrifuged at 8800 × *g* for 4 min. The supernatants were carefully pipetted off and discarded. The pellets were washed once by suspension in 0.75 ml of a 50% saturated ammonium sulfate solution and centrifugation at 8800 × *g* for 4 min. The supernatants were again discarded, and the tubes containing the pellets were counted in a gamma counter. The standard curves for cAMP (Fig. 1) obtained with the two forms of the radioimmunoassay were indistinguishable. Each sample was assayed in duplicate, and a complete standard curve for cAMP was included in each assay.

Phosphodiesterase treatments

Samples were treated with phosphodiesterase to destroy cAMP as follows. Aliquots (200 µl) of samples were each mixed with 20 µl of phosphodiesterase (~ 50 mU) as supplied. The samples were incubated for 1 h at 37 °C. The reaction was stopped by heating the samples at 100 °C for 5 min. The samples were then centrifuged at 8800 × *g* for 2 min and the supernatants assayed for cAMP by radioimmunoassay. Controls containing cAMP standards, but not phosphodiesterase, showed that cAMP was stable under these incubation conditions.

Results and Discussion

The use of a radioimmunoassay allowed the facile detection and quantitation of cAMP in extracts of soybean hypocotyls (Table I). The amount of cAMP detected in untreated soybean hypocotyls was low (0.6 pmol/g fr. wt.) when compared with amounts found in other plants [5]. However, these levels of cAMP are well within the detection limit of the radioimmunoassay (0.1 pmol/g fr. wt.) (Fig. 1). That the radioimmunoassay was truly measuring cAMP in the hypocotyl extracts is indicated by two criteria. First, the antibodies used in the radioimmunoassay are specific for cAMP [15]. Secondly, no cAMP could be detected in tissue extracts after treatment of the extracts with cyclic nucleotide phosphodiesterase. Further controls showed that the cAMP did not originate in the solvents used to extract the hypocotyls tissue.

The amount of cAMP present in soybean seedlings infected with *Phytophthora megasperma* f. sp. *glycinea* was measured at various times after infection and compared with the amount of cAMP found in unwounded and wounded plants (Table I). Börner and Grisebach [2] reported that the activities

Table I. Quantitation of cAMP in soybean hypocotyls infected with *Phytophthora megasperma* f. sp. *glycinea* (Race 1).

Experiment	Incubation Time [hours]	Treatment[a]		
		None	Wounding	Infection
		[fmol cAMP/hypocotyl]		
I	2	34	34	23
	4	31	33	38
	7	40	36	29
	18	32	31	38
II	0.5	41	21	35
	1.0	< 5	36	34
	2.0	46	25	40
	2.5	13	< 5	10
III	2	24	34	43
	3	35	29	46
	4	13	27	44
	5	51	39	26

[a] Data represent the average of 2 determinations. Ten hypocotyls were used per treatment.

of several enzymes involved in phytoalexin biosynthesis reach a maximum 15 h after infection with *P. megasperma*. Thus, if cAMP were to be involved in the signal cascade leading to the *de novo* synthesis of these enzymes, one would expect to observe a rise in cAMP levels in infected plants well before this time. We were unable to detect such an increase at any time after infection.

We observed considerable variation in the levels of cAMP in the hypocotyls irrespective of the treatment the hypocotyls received (Table I). For example, between 0.5 fmol/hypocotyl and 51 fmol/hypocotyl of cAMP were found in unwounded, uninfected hypocotyls, while infected seedlings contained between 11 fmol/hypocotyl and 46 fmol/hypocotyl. These fluctuations could not be correlated with infection. That is, the variation observed in cAMP levels amongst unwounded, uninfected seedlings was as great as the variation observed between infected and uninfected plants. Certainly, there was no consistent increase in cAMP content of the hypocotyls after infection. In fact, in some cases less cAMP was found in infected seedlings than in untreated ones. These results suggest that cAMP does not play a physiological role in the response of soybean seedlings to infection with *P. megasperma* f. sp. *glycinea*. In addition, the variation in cAMP levels could indicate that the cAMP found in the hypocotyls originates from microorganisms present in the plant tissue, rather than from the plant tissue itself.

The possibility remained that the inability to detect changes in cAMP levels in the soybean hypocotyls after infection was due to too few soybean cells being induced by the invading hyphae. To examine this possibility, soybean cell suspension cultures were treated with a glucan elicitor from *P. megasperma* which has previously been shown to induce enzymes of phytoalexin biosynthesis in such cultures [11, 14]. Since most or all cells in a culture so treated will come into contact with the elicitor, greater amounts of uniformly induced cells can be obtained from such an experiment than from infected hypocotyls. We were unable to detect any cAMP either in control (water-treated) or elicitor-treated soybean cell suspension cultures, even though phenylalanine ammonia-lyase was induced by the latter treatment. The detection limit for cAMP under the extraction conditions used was 75 fmol/g fr. wt. Exogenously added authentic cAMP could be recovered in the cell extracts, thus, we would have been able to detect cAMP had it been present in the cells. The inability to detect any cAMP in soybean cell suspension cultures in which the enzymes for phytoalexin biosynthesis were induced, implies that the induction of this pathway does not rely on cAMP as a second messenger. Furthermore, these data show that soybean cells are able to grow and remain healthy without producing any cAMP.

The finding that soybean cell suspension cultures produce no cAMP is in contrast to the work of Brewin and Northcote [16]. These authors were able to detect 2 to 40 pmol/fr. wt. of cAMP in soybean cell suspension cultures using a cAMP-binding protein assay. The reasons for this discrepancy are not clear. The extraction technique used in the present report does not differ substantially from those used by others [7, 16]. Furthermore, the radioimmunoassay would have easily detected such amounts of cAMP had they been present in our cultures.

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