Phytochemistry, Vol. 26, No. 7, pp. 1919–1923, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00

THE ELICITATION OF PHYTOALEXINS BY Ca^{2+} AND CYCLIC AMP IN CARROT CELLS

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(*Received* 21 *October* 1986)

Key Word Index—*Daucus carota*; Umbelliferae; calcium ion; cell culture; cyclic AMP; elicitor; phytoalexin.

Abstract—Addition of calcium ionophore A23187 or dibutyryl cyclic AMP (dBcAMP) to carrot (*Daucus carota* L.) cell culture induced the production of 6-methoxymellein, a phytoalexin of carrot, in a dose-dependent manner. Several reagents known to suppress the cytoplasmic calcium concentration appreciably inhibited elicitor-promoted phytoalexin production in carrot cells. The addition of elicitor to the carrot culture caused a rapid increase in the intracellular level of cyclic AMP. Treatments of the cells with theophylline or cholera toxin stimulated the biosynthesis of 6-methoxymellein even in the absence of elicitor. These observations suggested that Ca^{2+} and cyclic AMP participate as second messengers in the regulation of 6-methoxymellein production in cultured carrot cells. Addition of verapamil to carrot cell culture markedly inhibited 6-methoxymellein production when it was added within 30 min after elicitor-treatment of the cells, but no inhibitory effect was observed after 60 min. The results suggest that these messengers function in an early stage of the elicitation process. Carrot cells which were previously treated with verapamil accumulated only small amounts of 6-methoxymellein following the addition of dBcAMP. In contrast, cells incubated initially with dBcAMP accumulated the phytoalexin at levels comparable to the control when verapamil was added to the culture.

INTRODUCTION

The accumulation of phytoalexins in higher plants in response to microbial invasion is considered to be an important factor in the defense mechanisms of plants [1]. Production of these compounds is triggered by certain molecules of fungal origin [2], termed elicitors, and also by structurally unrelated compounds such as heavy metals [3, 4] and denatured RNase [5]. Recent investigations show that elicitor-active molecules are also released from the host plants [6–8]. These endogenous elicitors are thought to be liberated from host plants by the action of hydrolytic enzymes which are secreted from invading fungi or bacteria.

We reported previously [7] that the phytoalexin of carrot, 6-methoxymellein, was produced in suspension-cultured carrot cells when the cells were treated with pectinase. Proteolytic enzymes also gave similar results. Unlike the results reported for leguminous plants, fungal components other than these extracellular enzymes were not effective in stimulating phytoalexin accumulation in carrot [9, 10]. Recently we have shown [11] that elicitor-active molecules were released from the pectic fraction of cultured carrot cells by limited hydrolysis with pectinase or trypsin. These elicitors were partially purified and were found to be a heterogenous mixture of molecules in which uronide or peptide moieties are thought to be the essential structures. These results indicate that the phytoalexin production in carrot cells is elicited by a variety of wall components rather than a single substance. As the production of 6-methoxymellein in carrot cells can be regarded as a specific response of the cells, it seems strange that this reaction is induced by various molecules of different chemical species. Therefore, we suspected the possibility that second messengers, such as Ca^{2+} or cyclic AMP, or related factors may participate in, and regulate the elicitation process of the phytoalexin production in carrot. In the present experiments, we examined the effect of various reagents which are known to modulate the intracellular levels of Ca^{2+} and cyclic AMP on phytoalexin accumulation in cultured carrot cells. Related physiological changes of carrot cells occurring after the addition of elicitor were also studied.

RESULTS

Effect of calcium ionophore and dibutyryl cyclic AMP (dBcAMP) on 6-methoxymellein production in cultured carrot cells

6-Methoxymellein production in cultured carrot cells was induced when the cells were incubated with appropriate concentrations of calcium ionophore A23187 even in the absence of elicitor prepared from carrot cell homogenate (Fig. 1). The content of 6-methoxymellein increased in a dose-dependent manner, and attained a maximal value at about 0.5 μM of the ionophore. In higher concentrations, the accumulation of 6-methoxymellein apparently decreased. In the presence of the elicitor, the ionophore also stimulated the accumulation of the phytoalexin in carrot cells. In this case, the maximal accumulation of 6-methoxymellein was achieved at a lower concentration (0.1–0.3 μM) of the ionophore than in the absence of the elicitor. The maximum amounts of the compound, however, were comparable in both the presence and the absence of the elicitor.

Addition of dBcAMP to carrot cell culture also stimulated 6-methoxymellein production (Fig. 2). Maximal accumulation of the phytoalexin was observed at

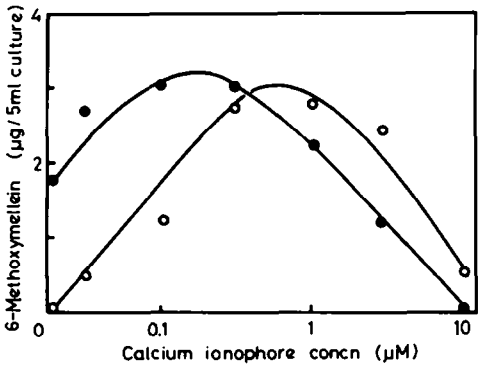

Fig. 1. 6-Methoxymellein production in cultured carrot cells treated with calcium ionophore A23187. The calcium ionophore (50 μl of ethanol solution) was added to 10-day-old carrot cell culture (5 ml) in the presence (●) or absence (○) of elicitor. 6-Methoxymellein was determined after a 24 hr incubation, and the results were expressed as means of four replicate experiments.

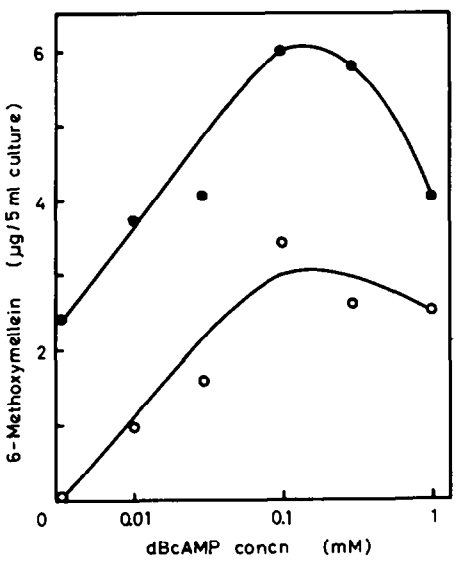

Fig. 2. 6-Methoxymellein production in cultured carrot cells treated with dBcAMP. dBcAMP (50 μl of ethanol solution) was added in the presence (●) or absence (○) of elicitor. 6-Methoxymellein was determined after 24 hr incubation, and the results were expressed as means of four replicate experiments.

ca 0.1–0.2 mM of dBcAMP. In the presence of both elicitor and dBcAMP, their effects were completely additive in the concentration range tested.

Effect of reagents that may influence the intracellular levels of Ca^{2+} *and cyclic AMP*

Effects of various reagents known in other systems to change the level of intracellular Ca^{2+} or cyclic AMP were examined in the presence or absence of the elicitor (Table 1). 6-Methoxymellein production was appreciably suppressed when the cells were transferred to Ca^{2+}-free medium shortly before the addition of elicitor. Viability and cell number after this treatment was examined under a microscope [12] and no appreciable difference was observed between control and treated cells (data not shown). Therefore, extra- and/or intra-cellular Ca^{2+} is considered to be an important factor in 6-methoxymellein production of cultured carrot cells. Addition of ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), a specific chelating agent for Ca^{2+}, to the elicitor-treated culture also gave similar results. Verapamil and [8-*N,N*-(dimethylamino)]octyl-3,4,5-trimethoxybenzoate (TMB-8) are well known compounds which suppress the increase of cytoplasmic level of Ca^{2+}. Both inhibitors appreciably inhibited the elicitor-promoted phytoalexin production in carrot cells.

Table 1. Effects of various treatments on the phytoalexin production of cultured carrot cells. The results were expressed as percentage of the amount of 6-methoxymellein accumulated in elicitor-treated cells. After being sterilized, 50 μl aliquots of the reagents were added to 5 ml of carrot cell culture (10-day-old) to give the final concentration indicated. Data were means of four replicate experiments

Reagents		6-Methoxymellein production (%)	
		+ Elicitor	− Elicitor
−Calcium*		33	0
EGTA†	(3 mM)	30	0
Verapamil‡	(50 μM)	18	0
TMB-8§	(0.2 mM)	13	0
Cholera toxin ¶	(1 μg/ml)	212	78
Theophylline†	(0.05 mM)	123	14
	(0.5 mM)	161	60
	(5 mM)	33	72
dBcGMP§	(0.3 mM)	81	0

*Prior to the addition of elicitor, cells were successively washed with Ca^{2+} free medium with and without 3 mM EGTA.

†Dissolved in Na-acetate buffer (0.1 M, pH 5.2), and sterilized by autoclaving.

‡Dissolved in water and sterilized by autoclaving.

§Dissolved in ethanol.

¶Dissolved in water and sterilized by filtering through a Millipore filter (0.22 μm).

Cholera toxin, an activator of adenylate cyclase, has an ability to induce the accumulation of 6-methoxymellein in cultured carrot cells in the absence of elicitor. When it was added to an elicitor-treated culture, the content of the phytoalexin was almost 2-fold higher than that of the culture treated with elicitor only. These results suggest that the elevation of intracellular level of cyclic AMP stimulates the initiation of the phytoalexin production in carrot cells. The idea is supported by the fact that the treatment of cultured carrot cells with theophylline, a phosphodiesterase inhibitor, also stimulated 6-methoxymellein production in the absence of the elicitor. Incubation of the cells with a relatively high concentration of theophylline (5 mM) and the elicitor, however, caused a decrease in the production of 6-methoxymellein. As seen in Fig. 2, an appropriate concentration of cyclic AMP is required to accumulate a high level of 6-methoxymellein in carrot cells. The addition of both the elicitor and phosphodiesterase inhibitor to the carrot cell culture might cause overaccumulation of intracellular cyclic

AMP to less effective levels. In contrast to the effect of dBcAMP, the addition of dibutyryl cyclic GMP (dBcGMP) did not stimulate phytoalexin production in cultured carrot cells.

Change of calcium flux and cyclic AMP level during phytoalexin production in cultured carrot cells

To confirm whether the intracellular levels of Ca^{2+} and cyclic AMP are actually raised by the addition of the elicitor, calcium flux into cytoplasm, cyclic AMP content and change in 6-methoxymellein content in the elicitor-treated carrot cells were examined with elicitor-treated cells. Since it is known that significant amounts of Ca^{2+} are localized in the plant cell wall, it is difficult to measure the flow of Ca^{2+} by means of fluorometric or radiochemical procedures. Instead, we employed an indirect method. After the elicitor was added to carrot cell culture, 50 μM of verapamil was added at various times during a 24 hr incubation to inhibit any calcium flux which may be enhanced by the action of elicitor.

When the elicitor was added to carrot suspension culture, the accumulation of 6-methoxymellein commenced after *ca* 8 hr, and then, the content increased with time (Fig. 3). Verapamil appreciably inhibited elicitor-promoted 6-methoxymellein production when it was added immediately after the addition of elicitor (Fig. 4). However, its inhibitory effect decreased rapidly as the timing of the addition of the reagent was delayed. After 40–60 min of induction, verapamil did not show any appreciable effect on 6-methoxymellein production in carrot cells. From these results, it is considered that the elicitor increased the cytoplasmic calcium level, which in turn induced the 6-methoxymellein production. An initial step which is stimulated by the flux of Ca^{2+} appeared to be terminated within 40–60 min after the addition of elicitor.

Intracellular cyclic AMP also increased after the addition of elicitor. The content usually reached a maximum value at 20–40 min incubation (Fig. 5) which was followed by a rapid decrease. In control culture without the addition of elicitor, on the other hand, the change in cyclic AMP content was negligible.

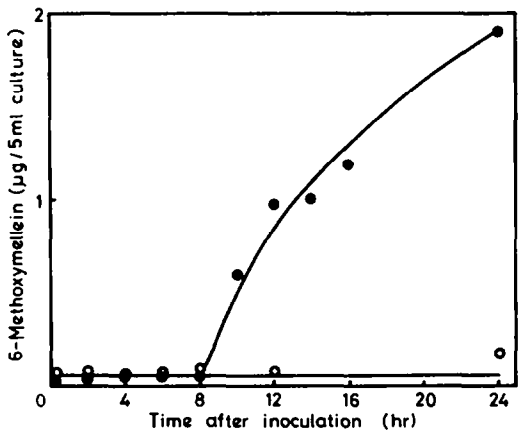

Fig. 3. Change in 6-methoxymellein content with time after elicitor addition in cultured carrot cells. Elicitor (200 μl) was added to cell culture (●), and 6-methoxymellein accumulated in the cells was determined at regular intervals. Control culture (○) received only Na-acetate buffer (0.1 M, pH 5.2).

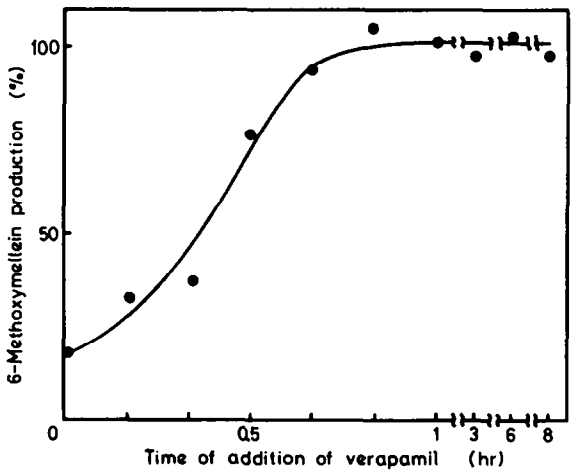

Fig. 4. Effect of addition of verapamil on 6-methoxymellein production in cultured carrot cells. A50 μl-aliquot of verapamil solution was added to elicitor-treated carrot cell culture (final concn 50 μM) at the time indicated on the abscissa. 6-Methoxymellein content in carrot cells was determined after 24 hr, and the results were expressed as the percentage compared to a control culture without verapamil. The data represent the means of four replicate experiments.

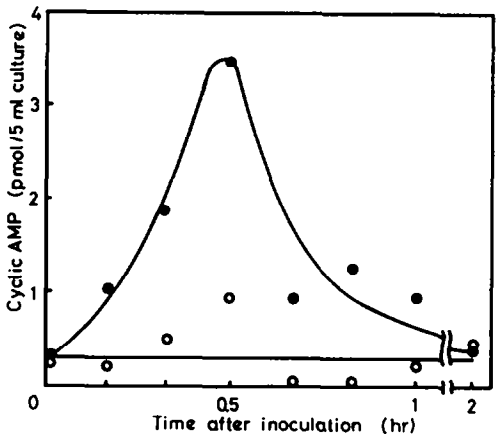

Fig. 5. Change in intracellular cyclic AMP level in cultured carrot cells. Elicitor was added to 10-day-old carrot cell culture, and the content of cyclic AMP in the cells was determined by radioimmunoassay (●). See Experimental for details. Control culture (○) received only Na-acetate buffer (0.1 M, pH 5.2).

Correlation of Ca^{2+} and cyclic AMP in elicitation process

Experiments were next undertaken to examine whether Ca^{2+} and cyclic AMP independently participate in elicitation process of 6-methoxymellein production, or whether these two messengers act correlatively with each other. The results shown in Table 2 indicate that carrot cells which were preincubated with verapamil for 1 hr accumulated only small amount of 6-methoxymellein, even though they were successively incubated with dBcAMP for 24 hr. By contrast, the cells incubated with dBcAMP first, had an ability to produce appreciable amount of the phytoalexin even though they were successively treated

Table 2. Effect of pretreatment with verapamil or dBcAMP on the phytoalexin production in cultured carrot cells. Data were means of four replicate experiments

First treatment (1 hr)	Second treatment (24 hr)	6-Methoxymellein (μg/5 ml culture)
—	Ethanol	0.32
Ethanol	—	0.23
—	dBcAMP	3.44
Ethanol	Verapamil	0.34
Verapamil	dBcAMP	1.41
dBcAMP	Verapamil	2.97

Carrot cells were pretreated with the first reagent for 1 hr, then, successively treated with the second reagents for 24 hr. Verapamil (final concn 50 μM) was dissolved in water, and dBcAMP (final concn 0.1 M) was in ethanol, and a 50 μl-aliquot of the solution was added to 5 ml of carrot cell culture.

with verapamil. These observations suggest that Ca^{2+} and cyclic AMP do not act as messengers independently, but that they are correlated with each other in the elicitation of phytoalexin production in cultured carrot cells.

DISCUSSION

Recently, many workers have tried to understand the host–parasite interactions at the molecular level, and special attention has been focused on the isolation, characterization and structure elucidation of elicitors which stimulate phytoalexin production in infected plants. These experimental results show that a wide variety of substances are able to act as elicitors [2]. The findings that the phytoalexin production is triggered by structurally unrelated substances raise the possibility of the participation of second messengers in the elicitation process of phytoalexin production. Although some reports have dealt with a possible role of second messengers in defense responses of plant cells, such as production of antivirulent factor and callose synthesis [13–16], the physiological significance of these messengers in phytoalexin production has not yet been documented.

The results presented here show that the treatment of cultured carrot cells with calcium ionophore A23187 (Fig. 1) and dBcAMP (Fig. 2), which cause the elevation of intracellular Ca^{2+} and cyclic AMP, respectively, induced the production of 6-methoxymellein in a dose-dependent manner. In addition, several reagents, which are known to suppress the increase of cytoplasmic Ca^{2+} concentration, were shown to inhibit the elicitor-promoted 6-methoxymellein production in cultured carrot cells (Table 1). Treatments with theophylline and cholera toxin, which cause the increase in the concentration of intracellular cyclic AMP, enhanced the biosynthesis of 6-methoxymellein. Furthermore, it was also confirmed that the addition of elicitor to the carrot cells led to the appreciable increase of cyclic AMP level in the cells (Fig. 5). These results strongly suggest that both Ca^{2+} and cyclic AMP play significant roles in the elicitation process of phytoalexin production in carrot cells. Hahn and Grisebach [17] determined cyclic AMP levels in *Phytophthora megasperma*-infected soybean hypocotyls, and in suspension cultured cells treated with glucan elicitor of the fungus. However, they could not find any correlation between infection and cyclic AMP levels in soybean cells. It is uncertain whether this discrepancy is due to the differences in the experimental conditions or if it is based on the inherent differences in the properties of these cells. By contrast, Rosenberg *et al.* [14] reported the change in cyclic AMP content in *Nicotiana glutinosa* L. infected by tobacco mosaic virus. Abad *et al.* [16] also showed that adenylate cyclase activity in *Nicotiana tabacum* leaves was enhanced by infection of the virus.

Although 6-methoxymellein accumulation was detected about 8 hr after the addition of elicitor, an initial step of the phytoalexin induction seemed to occur immediately after the addition of elicitor. When verapamil was added to the cell culture within 30 min of the elicitor-treatment, the accumulation of 6-methoxymellein was strongly inhibited (Fig. 4). The addition of elicitor to carrot cells resulted in a rapid accumulation of cyclic AMP which lasted about 30 min (Fig. 5). These observations suggest that these second messengers participate in 6-methoxymellein production in an early stage of the elicitation process.

Preincubation of carrot cells with verapamil appreciably inhibited dBcAMP-promoted 6-methoxymellein production (Table 2). On the other hand, the cells previously treated with dBcAMP accumulated significant amounts of the compound even though verapamil was added to the culture 1 hr after the addition of dBcAMP. Although the physiological function of cyclic AMP in higher plants is still obscure, it is conceivable from the results obtained with mammalian cells that cyclic AMP possibly affects 6-methoxymellein production by regulating a calcium gating mechanism of carrot cells. For example, Heisler and Reisine [18] reported that, in adrenocorticotropin secretion from mouse tumour cells, cyclic AMP synthesis may be linked to a distal mechanism which increases calcium flux into the cells. To elucidate the overall mechanism of elicitation process of phytoalexin production in cultured carrot cells, participation of other related message systems, such as turnover of phospholipids in cellular membrane or calmodulin-mediated physiological regulations, should be examined in detail.

EXPERIMENTAL

Carrot cell culture. Carrot cells (*Daucus carota* L. cv Kintoki) were grown on a reciprocal shaker (120 strokes/min) in the liquid medium of ref. [19] in the presence of 1 mg/l of 2,4-dichlorophenoxyacetic acid according to ref. [20].

Chemicals. Calcium ionophore A23187, dBcAMP, dBcGMP, verapamil, cholera toxin and TMB-8 were purchased from Sigma. EGTA and theophylline were from Nakarai Chemicals Ltd.

Production of 6-methoxymellein in cultured carrot cells. Crude elicitor was prepared by partial hydrolysis of carrot cells by the method of ref. [12]. Cultured carrot cells (7-day-old, 10 g fr. wt) were harvested by filtration and suspended in 25 ml of NaOAc buffer (0.1 mM, pH 5.2). The suspension was autoclaved to kill the cells and homogenized by sonication. After cooling, the homogenate was incubated with 300 units of pectinase (Sigma) for 3 hr at 37°. The reaction was terminated by boiling for 20 min, and the homogenate was centrifuged at 10000 *g* for 20 min. The resulting supernatant, which contained elicitor-active components, was sterilized by autoclaving, and a 200 μl-aliquot was added to 5 ml of carrot cell culture (10 days old). The cells were harvested after 24 hr incubation to determine the 6-methoxymellein content.

Determination of 6-methoxymellein. Procedures for the extraction and partial purification of 6-methoxymellein from cultured carrot cells were described in ref. [9]. Partially purified materials were developed on a TLC plate (silica gel 60 F 245, Merck) in C_6H_6–MeOH (50:1) and the amount of 6-methoxymellein was determined by scanning at 265 nm (ref. 400 nm) using a dual wavelength chromatoscanner. Data were expressed as the means of four replicate experiments. Standard deviations of the results were < 20%.

Determination of cyclic AMP. The amount of cyclic AMP in carrot cells was determined radioimmunologically [21] using a commercial assay kit (cyclic AMP [^{3}H] radioassay kit, Amersham). Carrot cells in 5 ml culture (*ca* 0.5 g) were harvested by filtration and immediately frozen in liquid N_2. The cells were mixed with cold 10% TCA (3 ml) and homogenized in a mortar with pestle. The homogenate was centrifuged at 10000 *g* for 20 min. To the supernatant soln, 1 drop of 6M HCl was added and it was shaken with 2 ml of Et_2O (× 6) to remove TCA. The aq. phase was collected by centrifugation, neutralized by two drops of 28% NH_4OH, and then, passed through an alumina column (5 × 80 mm, neutral alumina, Merck). The column was washed with 4 ml H_2O, and the eluent was lyophilized and redissolved in 150 μl of Tris–HCl buffer (50 mM, pH 7.5, containing 4 mM EDTA). Partially purified samples thus obtained were subjected to radioimmunoassay as described below. Recovery of the compound by this procedure was measured using [^{3}H]-labelled cyclic AMP, and found to be 95.1 ± 4.6% (mean and s.e. from 5 replicate experiments). Radioimmunoassay was performed according to the protocol of the assay kit. Sample soln (50 μl) was incubated with antibody at 0° for 2 hr, and separation of the protein bound cyclic AMP from the unbound nucleotide was achieved by adsorption of the free nucleotide on to coated charcoal. The radioactivity of the supernatant was measured by a liquid scintillation spectrometer and cyclic AMP content was estimated from a standard curve drawn prior for each experiment.

Acknowledgement—This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

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