Phytochemistry, Vol. 26, No. 1, pp 51–53, 1987.
Printed in Great Britain

0031-9422/87 $3.00 + 0.00

CARROT PHYTOALEXIN INHIBITS Ca^{2+}, CALMODULIN-DEPENDENT PROTEIN PHOSPHORYLATION IN CARROT CELLS

MAGDY AMIN, FUMIYA KUROSAKI and ARASUKE NISHI*

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan

(*Revised received* 28 *March* 1986)

Key Word Index—*Daucus carota*; Umbelliferae; carrot; phytoalexin; 6-methoxymellein; calmodulin; protein phosphorylation.

Abstract—Phosphorylation of soluble proteins obtained from cultured carrot cells was monitored by measuring the incorporation of ^{32}P from [^{32}P]ATP into the trichloroacetic acid insoluble fraction. The reaction was stimulated by Ca^{2+} and calmodulin, and inhibited by a carrot phytoalexin, 6-methoxymellein. 6-Methoxymellein also inhibited the Ca^{2+}, calmodulin-dependent phosphorylative activation of NAD:quinate oxidoreductase (EC 1.1.1.24) partially purified from the carrot cells. The inhibitory effect of 6-methoxymellein was reduced when the reaction mixture contained a high concentration of calmodulin.

INTRODUCTION

Carrot phytoalexin, 6-methoxymellein (6 MM), has been shown to display potential biological toxicity in a wide range of organisms including its host plant [1–3]. The mechanism by which 6 MM stops the growth of cells is not yet understood. Recently, we have shown that 6 MM inhibits the calmodulin activation of bovine cyclic nucleotide phosphodiesterase (PDE) [4]. Calmodulin is a ubiquitous protein of great biological significance and has been implicated in the Ca^{2+}-dependent regulation of many biological reactions [5]. The experiments reported here add further evidence that 6 MM inhibits the cellular processes by interfering with the Ca^{2+}-calmodulin system involved in their activation.

*To whom correspondence should be addressed.

RESULTS AND DISCUSSION

A soluble fraction of cultured carrot cells was examined for the activity of Ca^{2+}, calmodulin-dependent phosphorylation (Table 1). The phosphorylation started immediately after the addition of ATP, it proceeded in a linear fashion and reached a maximum after about 10 min. The reaction rate in the presence of Ca^{2+} was 1.5–2 times higher than that in its absence. The addition of bovine calmodulin to the assay mixture in the presence of Ca^{2+} obviously enhanced the phosphorylation. These results indicate that Ca^{2+} and calmodulin regulate the phosphorylation of soluble proteins in the carrot cells. These findings are analogous to those reported previously for protein phosphorylation in the isolated membranes of pea shoots [6] and zucchini hypocotyls [7] and the soluble proteins from corn [8] and *Neurospora crassa* [9].

6 MM inhibits the calmodulin activation of bovine PDE [4]. When 6 MM was added to the assay mixture of

Table 1. Effect of calmodulin concentration on the inhibition of Ca^{2+}-dependent protein phosphorylation by 6MM

Additions	Radioactivity (cpm/mg protein) Expt 1	Expt 2
None (control)	1017	924
Ca^{2+} (1 mM)	1526	1510
Ca^{2+} + calmodulin (0.6 μg/ml)	2231	2418
Ca^{2+} + calmodulin (5.6 μg/ml)	4557	4912
Ca^{2+} + calmodulin (0.6 μg/ml) + 6MM (100 μM)	926	1006
Ca^{2+} + calmodulin (5.6 μg/ml) + 6MM (100 μM)	4116	4018

Phosphorylation of protein was initiated by the addition of [γ-^{32}P]ATP and terminated after incubation at 30° for 7.5 min by adding 3 volumes of 12% TCA. The reaction mixture in the control run contained 0.2 mM EGTA. The radiolabelled proteins were separated and their radioactivities were measured as described in the Experimental.

protein phosphorylation, the Ca^{2+}-dependent phosphorylations were inhibited by the phytoalexin at concentrations comparable to the inhibitory range of a calmodulin antagonist, trifluoperazine (TFP) (Fig. 1). The apparent half-maximal inhibition was obtained at about 20-30 μm 6 MM under the present assay conditions. At the same concentration range of the phytoalexin, Ca^{2+}-independent phosphorylation was only slightly affected. The inhibitory effect of 6 MM on the phosphorylation reaction was, however, largely dependent on the concentration of calmodulin and was greatly reduced when the assay mixture contained a high concentration of this regulatory protein (Table 1). These results suggest that 6 MM inhibits Ca^{2+}, calmodulin-dependent activation of protein phosphorylation.

In carrot, NAD:quinate oxidoreductase (QORase) has been shown to undergo deactivation by Mg^{2+}-dependent dephosphorylation and reactivation by Ca^{2+}, calmodulin-dependent phosphorylation. The enzyme activity is strictly correlated to the degree of phosphorylation and the QORase activity could be taken as a measure of protein phosphorylation [10]. 6 MM inhibited the phosphorylative reactivation of QORase in a concentration-dependent fashion (Fig. 2). Since 6 MM (50 μM) had no effect on the inactivation process of QORase (data not shown), it appeared specifically to inhibit the Ca^{2+}, calmodulin-dependent phosphorylation of the enzyme. This view was confirmed by the results of the experiment shown in Fig. 3. The addition of bovine brain calmodulin to the assay mixture of QORase resulted in a decrease in the inhibitory effect of 6 MM.

We previously reported that 6 MM at a concentration above 100 μm completely inhibits the calmodulin-dependent activation of bovine PDE [4]. In the present experiment, it is shown that Ca^{2+}, calmodulin-dependent protein phosphorylation in plants may also be completely inhibited at the same concentration of the phytoalexin. Since a recent report has demonstrated the presence of a Ca^{2+}, calmodulin-dependent protein kinase in *Neurospora crassa*, it may be considered that 6 MM exerts toxic effects on various fungi in a similar way. It is likely that calmodulin is a target for the toxicity of carrot phytoalexin in eukaryotes, although other targets cannot be excluded.

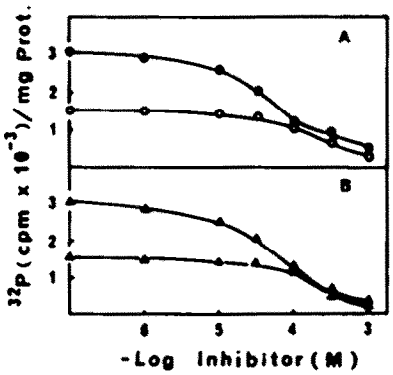

Fig. 1. Effect of 6 MM (A) TFP (B) on Ca^{2+}-dependent (●, ▲) and independent (○, △) phosphorylation of soluble protein from carrot cells. 6 MM was dissolved in ethanol (final concentration 2%). The control run in the absence of 6 MM contained the same concentration of ethanol.

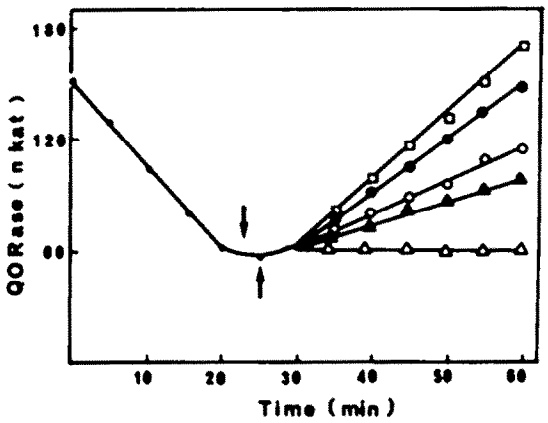

Fig. 2. Effect of 6 MM on the phosphorylative reactivation of QORase of carrot cells. The enzyme was first inactivated by incubating the assay mixture at 25° in the presence of 1 mM $MgCl_2$ for 20 min. 6 MM was added to the mixture at the time indicated by the arrow: □, 0; ●, 10; ○, 25; ▲, 50; △, 100 μM. Reactivation was initiated at the time indicated by the upward arrow by the addition of 4 mM ATP, 50 mM NaF and 1 mM $CaCl_2$. Enzyme activity at the indicated time was determined by measuring the difference in A at 340 nm in 2 min. In this experiment, 6 MM was dissolved in DMSO (final concentration) 0.05%).

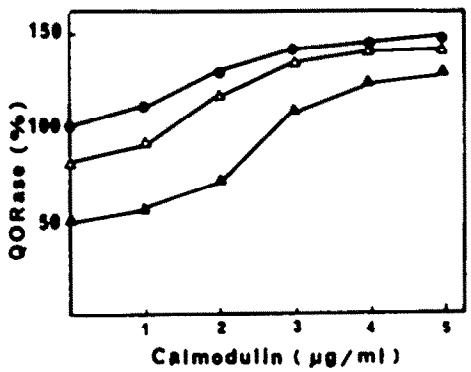

Fig. 3. Effect of calmodulin concentration on the inhibition of phosphorylative reactivation of QORase by 6 MM. 6 MM (●, 0; △, 10; ▲, 25 μM) and calmodulin were added to the reaction mixture containing partially inactivated QORase. Reactivation of the enzyme was performed as in Fig. 2. Data are expressed in percentage to the QORase activity obtained in the absence of both 6 MM and calmodulin.

EXPERIMENTAL

Isolation and purification of 6 MM. 6 MM was isolated and purified from carrot root slices infected by the fungus *Chaetomium globosum*, as described in ref. [1].

Preparation of carrot soluble proteins. Carrot cells were cultured in Murashige and Skoog's synthetic medium [11] as described in ref. [12]. 6-Day-old carrot cells were harvested by filtration and rapidly frozen in liquid N_2. The frozen cells were homogenized in a mortar with buffer A, containing 50 mM Tris–HCl (pH 7.8), 1 mM $MgSO_4$, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.25 M sucrose and 0.2 mM EGTA. The homogenate was centrifuged at

7000 *g* for 15 min to remove cellular fragments. The supernatant obtained after 105000 *g* centrifugation was dialysed against buffer B, containing 50 mM Tris–HCl (pH 7.8), 1 mM $MgSO_4$, 0.1 mM DTT and 0.2 mM EGTA. All procedures were done at 0–4°. The dialysed extract was adjusted to 5 mg protein/ml and stored at −30°. Care was taken to avoid repeated thawing and freezing, which results in loss of enzyme activity.

Assay of protein phosphorylation. Protein phosphorylation was measured by following the incorporation of ^{32}P from [^{32}P]ATP into TCA and EtOH insoluble fraction [13]. The standard assay mixture (0.5 ml) contained 50 mM Tris-HCl (pH 7.8), 1 mM $MgSO_4$, 0.1 mM DTT, 0.2 mM ATP (0.5–0.25 μCi [γ-^{32}P]-ATP, 0.5 mg carrot protein and 0.2 mM EGTA (for assay in the absence of Ca^{2+}) or 1 mM $CaCl_2$ (for assay in the presence of Ca^{2+}). The reaction was initiated by the addition of ATP and terminated after incubation at 30° for 7.5 min by the addition of 3 vols. of 12% TCA. The ^{32}P-labelled proteins were recovered as described in ref. [13] and their radioactivity was measured by a liquid scintillation spectrometer using a commercial scintillation mixture (Amersham ASC II).

Partial purification and assay of carrot QORase. Proteins were extracted from 6-day-old carrot cells and QORase was partially purified as in ref. [14]. The activity of QORase was determined at 25° by measuring the change in *A* at 340 mm due to NAD reduction based on the procedure described in ref. [15]. The standard assay mixture contained 0.5 M Tris HCl (pH 8.5), 0.2 M quinic acid, 2 mM NAD and 0.5 ml of the partially purified enzyme in a total vol. of 2 ml. Controls were run as above except for the absence of quinic acid. One unit of the enzyme catalyses the conversion of 1 mol of quinic acid per sec. Protein was determined by the method of ref. [16].

Chemicals. Bovine brain calmodulin and NAD were obtained from Sigma. [γ-^{32}P]ATP (28.8 Ci/mmol) was purchased from New England Nuclear. Trifluoperazine dimaleate (TFP) was obtained from Wako, ATP from Boehringer, and quinic acid from Aldrich.

REFERENCES

1. Kurosaki, F. and Nishi, A. (1983) *Phytochemistry* **22**, 669.
2. Coxon, D. T., Curtis, F. R., Price, K. R. and Levett, G. (1973) *Phytochemistry* **12**, 1881.
3. Kurosaki, F., Matsui, K. and Nishi, A. (1984) *Physiol. Plant Pathol.* **25**, 313.
4. Amin, M., Kurosaki, F. and Nishi, A. (1986) *Phytochemistry* **25**, 2305.
5. Manalan, A. S. and Klee, C. B. (1984) *Adv. Cyclic Nucleotide Protein Phosphoryl. Res.* **18**, 227.
6. Hetherington, A. and Trewavas, A. (1982) *FEBS Letters* **145**, 67.
7. Salimath, B. P. and Marmé, D. (1983) *Planta* **158**, 560.
8. Paliyath, G. and Poovaiah, B. W. (1985) *Plant Cell Physiol.* **26**, 201.
9. van Tuinen, D., Ortega Perez, R., Marmé, D. and Turian, G. (1984) *FEBS Letters* **176**, 317.
10. Ranjeva, R., Refeno, G., Boudet, A. M. and Marmé, D. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5222.
11. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.
12. Okamura, S., Sueki, K. and Nishi, A. (1975) *Physiol. Plant.* **33**, 251.
13. Soll, J. and Buchanan, B. B. (1983) *J. Biol. Chem.* **258**, 6686.
14. Refeno, G., Ranjeva, R. and Boudet, A. M. (1982) *Planta* **154**, 193.
15. Graziana, A., Boudet, A. and Boudet, A. M. (1980) *Plant Cell Physiol.* **21**, 1163.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.