Phytochemistry, Vol. 25, No. 10, pp. 2305–2307, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

INHIBITION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE BY CARROT PHYTOALEXIN

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(*Revised received* 6 *March* 1986)

Key Word Index—*Daucus carota*; Umbelliferae; phytoalexin; 6-methoxymellein; phosphodiesterase; calmodulin; metal cations.

Abstract—The carrot phytoalexin, 6-methoxymellein, was isolated and purified from carrot root slices infected by the fungus *Chaetomium globosum*. It inhibited the basal and calmodulin-promoted activity of cyclic nucleotide phosphodiesterase. The inhibition of calmodulin-promoted diesterase activity was reduced by increasing the concentration of calmodulin or calcium while the inhibition of basal diesterase activity was reversed by the addition of magnesium to the assay mixture of the enzyme.

INTRODUCTION

The mechanism of action of phytoalexins is not fully understood though some evidence suggests that they alter the plasma membrane [1, 2]. It has also been suggested that some phytoalexins inhibit electron transport in the mitochondria [3, 4]. The carrot phytoalexin 6-methoxymellein has been shown to inhibit the growth of bacteria, yeasts and fungi [5]. It is also toxic to animal [6] and plant cells [7]. Although the mechanism by which it inhibits the growth of cells has not been investigated, we have found that this phytoalexin interferes with cyclic nucleotide phosophodiesterase (PDE). The experiments reported here describe the effect of this phytoalexin on the activity of calmodulin-dependent PDE. The biological activity of the phytoalexin is discussed on the light of these results.

RESULTS AND DISCUSSION

In Fig. 1, evidence is presented that 6-methoxymellein inhibits the calmodulin-stimulated activity of PDE. The half-maximal inhibition of the enzyme activity occurred at a concentration of about 10^{-5} M. The inhibitory activity was comparable to that of trifluoperazine (TFP) which is a well-known inhibitor of calmodulin [8]. Unlike TFP, however, the inhibition was not restricted to the calmodulin-promoted PDE. When 6-methoxymellein was added to the reaction mixture at a concentration higher than 10^{-4} M, it also inhibits the basal enzyme activity in the absence of calmodulin. Increasing the concentration of calmodulin in the assay mixture overcame its inhibitory effect (Fig. 2). Similar effect of calmodulin concentration has been reported for other inhibitors of calmodulin-stimulated PDE [9, 10].

As shown in Fig. 1, 6-methoxymellein inhibited the basal activity of PDE. Since it has no structural similarity to the substrate cAMP, it is possible that this inhibition resulted from its interaction with a factor other than calmodulin and cAMP. The metal cation, Mg^{2+}, has been reported as an essential factor for the enzyme activity [11]. 6-Methoxymellein contains a hydroxyl and a carbonyl group in the molecule which are situated in the *peri* position so that it may bind to this metal cation. The results illustrated in Fig. 3 show that the inhibition of the basal activity of PDE by this phytoalexin is largely dependent on the concentration of Mg^{2+} added in the assay mixture. In the control run without 6-methoxymellein, PDE was fully activated by Mg^{2+} at a concentration lower than 1 mM. These results suggest that it inhibits the basal enzyme activity by causing magnesium deficiency in the reaction mixture. In this experiment, however, no attempt was made to examine if it could form a complex with Mg^{2+} ion. It may interact with other divalent metal cations including Ca^{2+}, which is involved in calmodulin-mediated activation of PDE. Figure 4

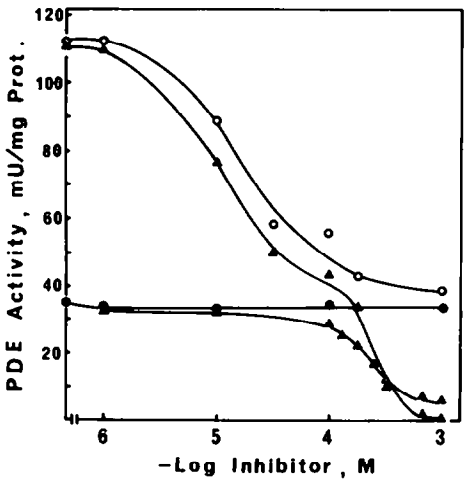

Fig. 1. Effect of 6-methoxymellein (△,▲) and trifluoperazine (○, ●) on cAMP phosphodiesterase from bovine heart. Open and closed symbols represent calmodulin-stimulated and basal enzyme activities, respectively. Each point represents the mean of six determinations.

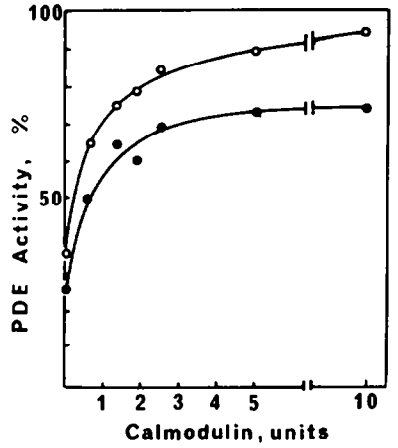

Fig. 2. Effect of calmodulin concentration on the inhibition of phosphodiesterase by 6-methoxymellein. The enzyme activity is expressed as % of control run in its absence. (○), 10^{-5} M; (●), 10^{-4} M. One unit calmodulin is 28 ng. Each point represents the mean of six determinations.

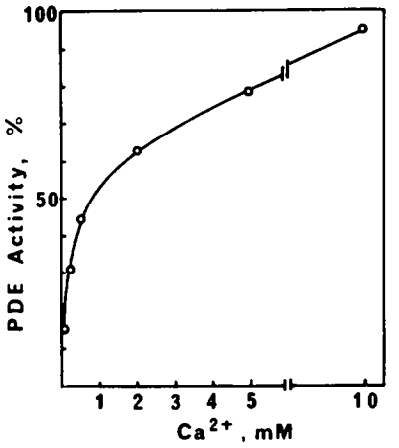

Fig. 4. Effect of Ca^{2+} concentration on the inhibition of calmodulin-stimulated activity of phosphodiesterase by 0.5 mM 6-methoxymellein. The enzyme activity is expressed as % of control run in the absence of phytoalexin. Each point represents the mean of six determinations.

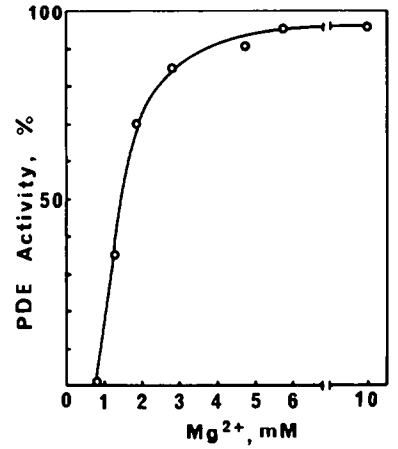

Fig. 3. Effect of Mg^{2+} concentration on the inhibition of basal phosphodiesterase activity by 2mM 6-methoxymellein. The enzyme activity is expressed as % of control run in the absence of phytoalexin. Each point represents the mean of six determinations.

shows that the inhibition of calmodulin-promoted PDE by 6-methoxymellein also depends on the concentration of Ca^{2+}. In its absence, maximum activation of PDE by calmodulin was achieved by the addition of 0.02 mM Ca^{2+} in the presence of 0.8 mM Mg^{2+}. When Ca^{2+} concentration was increased, its inhibitory effect was markedly decreased. Several calmodulin-antagonists exert their effect on calmodulin-mediated reactions by a similar mechanism [12].

Our results suggest that calmodulin is one of the target molecules for the toxicity of the carrot phytoalexin. Calmodulin, a ubiquitous protein of great biological significance, has been implicated in the Ca^{2+}-dependent regulation of many cellular processes. 6-Methoxymellein exerts a toxic effect on various fungi and also on plant cells at a concentration higher than 10^{-4} M which corresponds to that required for almost complete inhibition of bovine calmodulin-stimulated PDE activity though no experiment was performed with plant or fungal calmodulin.

It also seems likely that 6-methoxymellein interacts with metal cations and may inhibit enzymes activated by these metals. Its growth inhibition of prokaryotic cells could result from such a metal binding property. The elucidation of the full spectrum of its activity, however, requires further study.

EXPERIMENTAL

Isolation and purification of 6-methoxymellein. It was isolated from carrot root slices infected by the fungus, *Chaetomium globosum*, and purified as previously described [5].

Assay of cyclic nucleotide PDE. Assay of PDE was based on the method of Thompson and Appleman [13] using [^{3}H]cAMP. The assay was carried out at pH 7.5 and 37°. Unless specified otherwise, the reaction mixture (0.5 ml) contained 80 mM Tris–HCl buffer (pH 7.5), 0.02 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.1 mM cAMP, 50 ng calmodulin and 25 μg activator-deficient bovine heart PDE. 6-Methoxymellein was dissolved in EtOH giving a final concn of 2% EtOH in the assay mixture. The control always contained the same amount of EtOH. The reaction was started by the addition of the enzyme and terminated after 20 min by boiling for 3 min. Unhydrolysed cAMP was separated from the reaction product as in ref. [14] and the radioactivity of unhydrolysed [^{3}H]cAMP was measured by a liquid scintillation spectrometer using a commercial scintillation mixture (Amersham ACS II).

Chemicals. Bovine brain calmodulin, bovine heart activator-deficient PDE (EC 3.1.4.17) and cAMP were obtained from Sigma Chemicals Co. [2,8-^{3}H]cAMP (30.3 Ci/mmol) was purchased from New England Nuclear. Trifluoperazin dimaleate (TFP) was obtained from Wako Pure Chemicals Co., Japan.

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