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PHOSPHODIESTERASE ISOENZYMES IN CELL EXTRACTS OF CULTURED CARROT

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Key Word Index—*Daucus carota*; Umbelliferae; signal transduction; cyclic AMP; Ca^{2+}; phosphodiesterase; calmodulin; isoenzyme.

Abstract—Constitutive phosphodiesterase (PDE) activity found in cultured carrot cells did not depend on either Ca^{2+} or calmodulin (CAM), however, a CAM-dependent isoform of PDE (CAM-PDE) was induced in the cells by the addition of dibutyryl cAMP to the culture which elevates both cAMP and Ca^{2+} levels of cytosol. Induction of CAM-PDE activity in dibutyryl cAMP-treated carrot cells was markedly inhibited in the presence of a Ca^{2+} channel blocker, verapamil, and addition of Ca^{2+}-ionophore A23187 into the cell culture resulted in the induction of CAM-PDE. These observations suggest that increased Ca^{2+} but not cAMP in the stimulated carrot cells triggers the induction of the PDE isoenzyme. Affinity of CAM-PDE to the substrate was quite low as compared with the constitutive PDE (K_m values, 0.14 and 0.07 μM, respectively), however, V of the induced PDE was *ca* 2.7-fold higher than the constitutive isoenzyme. These results suggest that the constitutive PDE plays an important role in the maintenance of the resting state of carrot cells by keeping cellular cAMP and Ca^{2+} levels very low, while CAM-PDE induced in the excited cells hydrolyses the messenger nucleotide rapidly under the conditions of high cAMP and Ca^{2+} *in vivo*, as one of the response–decay mechanisms.

INTRODUCTION

The physiological functions of cAMP in higher plant cells are not well understood [1–4], however, we have demonstrated [5] that a unique signal-crosstalking of cAMP with Ca^{2+}-cascade is one of the mechanisms by which the nucleotide plays a role as a second messenger in the signal transduction responses of cultured carrot cells. Cytosolic concentration of cAMP transiently rises when the cells are appropriately stimulated, and the increased level of cAMP activates a Ca^{2+}-channel or the related structure at the plasma membrane without accompanying cAMP-dependent protein phosphorylation (Fig. 1). Cytoplasmic Ca^{2+} concentration reaches a level high enough to activate several Ca^{2+}-and/or calmodulin (CAM)-dependent processes, and appropriate cellular responses are evoked. Therefore, increase in cytosolic cAMP elicits Ca^{2+}-influx across the plasma membrane of carrot which is followed by the activation of Ca^{2+}-cascade in the cells. A similar mechanism of transmembrane signalling was reported in the odour-sensitive cells of a wide variety of animals [6–8]. We have recently shown [9] that several cellular events involved in the decay mechanisms of the cAMP-elicited responses in cultured carrot cells are also strictly controlled by cytoplasmic Ca^{2+} concentration (Fig. 1). It has been demonstrated [9] that adenylate cyclase activity was induced in carrot cells without a notable lag by the treatment with forskolin, an activator of the enzyme. The cyclase activity is highly sensitive to Ca^{2+} concentration, and is markedly inhibited in the presence of the ion above 0.5 μM [9]. Therefore, it is very likely that the cyclase is capable of catalysing cAMP synthesis only in the cells at the resting state, *in vivo*, and the activity is automatically diminished in the excited carrot cells in response to the increase in cytoplasmic Ca^{2+} level. It has been shown [9] that phosphodiesterase (PDE) activity in cultured carrot cells is also influenced by cytosolic Ca^{2+} level. Although the activity of the constitutive PDE found in carrot cells at the steady state did not depend on either Ca^{2+} or CAM, it appeared that a CAM-dependent isoform of PDE (CAM-PDE) was induced in forskolin-treated cells. Unlike in adenylate cyclase, however, CAM-PDE activity was found in the cells after a short lag period (3–5 min) of forskolin treatment, suggesting that the induction of the PDE isoenzyme is not a direct effect of forskolin but is rather a common cellular event responding to the increase in cAMP and/or Ca^{2+} levels in excited carrot cells. Although there have been several reports which described the biochemical properties of plant PDEs [10–12], only little is known about the induction and

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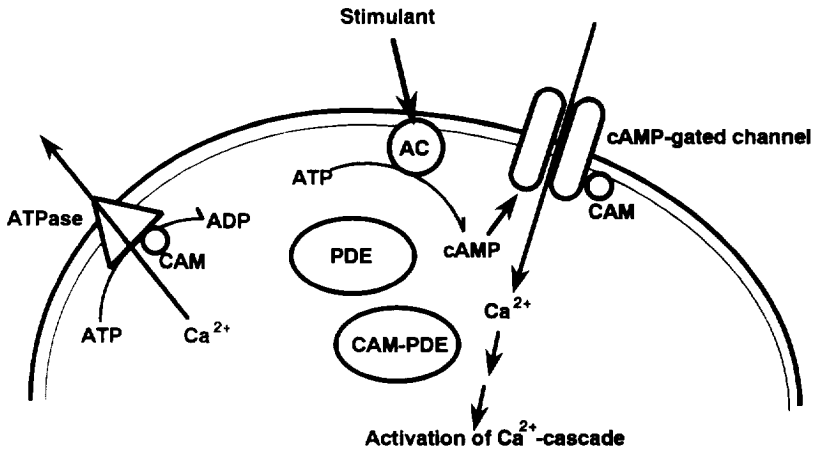

Fig. 1. Schematic presentation of cAMP-stimulated responses in cultured carrot cells. Cytoplasmic cAMP is maintained at a very low level by the action of constitutive PDE in the cells at the resting state. When adenylate cyclase (AC) is stimulated, elevated level of cAMP activates the Ca^{2+}-channel or the related structure at the plasma membrane. Increased Ca^{2+} activates the Ca^{2+}-cascade followed by the initiation of appropriate cellular responses, however, the messenger cations also function in the response–decay mechanisms. Elevated level of Ca^{2+} inhibits AC activity to stop the synthesis of cAMP, and induces CAM-PDE for the rapid hydrolysis of the messenger nucleotide. Activated CAM also plays roles in the closing mechanisms of cAMP-gated Ca^{2+}-channel to terminate the Ca^{2+}-influx, and in the enhancement of Ca^{2+}-ATPase activity for the translocation of the ions to cell exterior.

physiological difference of PDE isoenzymes. In the present experiments, we attempted to identify the cellular factor(s) responsible for the trigger of CAM-PDE induction. Possible different roles between these two PDE isoforms in cAMP-elicited signal transducing events in carrot cells is also discussed on the basis of the comparison of kinetic parameters of the isoenzymes.

RESULTS AND DISCUSSION

Induction of CAM-PDE in cultured carrot cells

We reported previously [5] that forskolin and dibutyryl cAMP (Bt_2cAMP) showed the same effects on the cytoplasmic levels of cAMP and Ca^{2+} in cultured carrot cells, and the addition of these reagents to the culture resulted in the increase in cellular concentrations of both messengers (Fig. 1). If the induction of CAM-PDE is triggered by the increase in cAMP and/or Ca^{2+} levels in carrot cells but not a direct effect of forskolin, treatment of the cells with Bt_2cAMP should induce the isoenzyme of PDE as observed in forskolin. As shown in Table 1, PDE activities in non-treated control cells assayed in the absence and presence of CAM showed almost the same values. In Bt_2cAMP-treated carrot cells, apparent PDE activity was similar to that of control when it was determined in the absence of CAM. However, the activity appreciably increased upon the addition of CAM into the assay mixture. These observations indicate that the CAM-dependent isoform of PDE was induced in cultured carrot cells by the treatment with Bt_2cAMP. Therefore, it is very likely that the induction of the PDE isoenzyme in carrot cells is not a specific effect of forskolin treatment, but is a common cellular response evoked by the increased level of the second messenger(s) in the excited cells. When cultured carrot cells were preincubated with a Ca^{2+} channel blocker, verapamil, the induction of CAM-PDE by Bt_2cAMP treatment was appreciably inhibited. This result suggests that the elevation of Ca^{2+} is an essential requirement for the induction of the PDE isoenzyme. To confirm this further, carrot cells were incubated with Ca^{2+}-ionophore A23187 in order to elevate cytoplasmic Ca^{2+} level specifically. Although constitutive PDE activity determined without the addition of CAM did not show any significant change as compared with the control, a marked increase in the enzyme activity was observed when the assay was carried out in the presence of CAM. These observations strongly suggest that the elevation of cytoplasmic Ca^{2+} concentration but not cAMP is the trigger event which elicits the induction of CAM-PDE in cultured carrot cells. CAM-dependent activity of PDE induced with Ca^{2+}-ionophore was, however, almost completely lost in the presence of 4 μM cycloheximide suggesting that *de novo* protein synthesis is necessary for the induction of the PDE isoenzyme.

Comparison of kinetic parameters of PDE isoenzymes

In the next experiments, kinetic parameters of these two PDEs were determined in order to understand possible different functions between these isoenzymes. PDE preparation was incubated with various concentrations of [^{3}H]cAMP in the absence or presence of CAM, and the results were analysed by a double reciprocal plot using the least squares method. Both PDE isoenzymes showed linear lines within the concentration range of 0.02–0.2 μM, and, as shown in Table 2, affinity of the

Table 1. Induction of PDE activities in cultured carrot cells by the treatment with Bt_2cAMP and Ca^{2+}-ionophore A23187

Treatments	PDE activity (pkat mg^{-1} protein)*			
	Experiment 1		Experiment 2	
	− CAM	+ CAM	− CAM	+ CAM
Control	0.27	0.24	0.31	0.25
Bt_2cAMP (100 μM)	0.25	0.62	0.23	0.85
Bt_2cAMP + verapamil (500 μM)	0.22	0.32	0.20	0.41
A23187 (1 μM)	0.28	0.81	0.33	0.70
A23187 + cycloheximide (4 μM)	0.24	0.26	0.29	0.21

*Cultured carrot cells (10-days-old) were incubated with Bt_2cAMP or Ca^{2+}-ionophore A23187 for 13 min, and PDE activity in the cell extracts was assayed in the presence of EGTA (− CAM) or Ca^{2+}/CAM (+ CAM). In some experiments, carrot cultures were preincubated with verapamil or cycloheximide (5 min) prior to the addition of the inducers.

Table 2. Kinetic parameters of PDE isoenzymes and adenylate cyclase in cultured carrot cells

Enzymes	K_m (μM)	V (pkat mg^{-1} protein)
PDEs		
Constitutive PDE	0.07	1.2
CAM-PDE	0.14	3.2
Adenylate cyclase	0.8	3.9

constitutive PDE towards its substrate was found to be considerably high as compared with CAM-PDE (K_m, 0.07 and 0.14 μM, respectively). By contrast, V of the constitutive PDE was appreciably lower than that of CAM-PDE (1.2 and 3.2 pkat mg^{-1} protein, respectively, under the present experimental conditions). It seems that these differences of the kinetic parameters between the two PDEs reflect the different functions of the respective isoenzymes. Constitutive PDE keeps cAMP and Ca^{2+} levels of carrot cytoplasm very low to maintain the resting state of the cells. Therefore, the K_m value of the isoenzyme should be relatively low to meet the cellular conditions of low cAMP level while hydrolytic rate toward the nucleotide might not be required to be high. CAM-PDE is induced in carrot cells in the excitatory state in order to hydrolyse cAMP of high concentrations rapidly. Therefore, its catalytic rate should be sufficiently high even if the affinity for the substrate could be quite low.

Adenylate cyclase activity was induced in carrot cells by the treatment with forskolin [9], and an attempt was made to compare the catalytic rates of biosynthesis and degradation of cAMP in stimulated carrot cells. The cyclase was prepared employing the same procedure for the preparation of PDEs, and only the components of homogenization buffer were modified to adapt to the characteristics of the respective enzymes. Therefore, V values estimated for these enzymes should roughly reflect the relative velocities of the catalytic reactions of synthesis and degradation of cAMP. As shown in Table 2, V of adenylate cyclase was similar to that of CAM-PDE. Therefore the catalytic rates of the biosynthesis and 'acute' degradation of cAMP in excited carrot cells were almost comparable. It seemed that the affinity of adenylate cyclase for its substrate ATP was also relatively high (K_m, 0.8 μM) as compared with other enzymes which share ATP as the common substrate. For example, the K_m value of an ATP-consuming enzyme at carrot plasma membrane relating transmembrane signalling, Ca^{2+}-pumping ATPase [13], was more than 200 μM (unpublished result).

Recently, a correlation of cyclic GMP and Ca^{2+} in the phytochrome phototransduction processes in soybean cell culture was reported [14]. However, as reported previously [15], addition of dibutyryl cyclic GMP to carrot cell culture had shown no significant effect in our experimental system. Possible interaction between cyclic GMP and Ca^{2+}-cascade, and role of PDEs in the metabolism of the nucleotide in carrot cells is therefore obscure at present. We showed [9] that adenylate cyclase in carrot cells is strongly inhibited by the elevated level of cytoplasmic Ca^{2+} in excited carrot cells of which influx is triggered by the product of the enzyme, cAMP, itself. In addition, we have reported previously [16] that plasma membrane-embedded CAM works as an essential component in the closing mechanisms of the cAMP-gated Ca^{2+}-channel to terminate the Ca^{2+} influx. More recently, we have demonstrated [13] that the activity of Ca^{2+}-pumping ATPase at carrot plasma membrane, a key component to maintain the cytoplasmic Ca^{2+} level low by mediating Ca^{2+} efflux, is also CAM-dependent. The present finding that the increase in Ca^{2+} level induces CAM-PDE with a high hydrolytic rate, together with

our previous reports, indicates that Ca^{2+} introduced in carrot cytosol by the action of cAMP plays important roles not only in the activation of Ca^{2+}-cascade but also in the response-decay mechanisms which restore the excited cells to the steady state.

EXPERIMENTAL

Chemicals. ATP, 5′-AMP, cAMP, creatine kinase, creatine phosphate and myokinase were purchased from Boehringer Mannheim. Verapamil, Ca^{2+}-ionophore A23187, bovine brain CAM and Bt_2cAMP were obtained from Sigma, while adenosine, EGTA, cycloheximide and 3-isobutyl-1-methylisoxanthine were from Wako. [^{3}H]cAMP (sp. act. 1.2 TBq mmol^{-1}) and [^{3}H]ATP (sp. act. 1.2 TBq mmol^{-1}) were obtained from New England Nuclear.

Induction and assay of CAM-PDE activity in cultured carrot cells. Cultured carrot cells were grown in 100 ml of Murashige-Skoog's synthetic medium [17] on a Innova 2300 rotary shaker (200 rpm) as described previously [13]. Induction of CAM-PDE activity in cultured carrot cells was carried out by the treatment of the cells with Bt_2cAMP or Ca^{2+}-ionophore A23187. Ten ml culture of carrot cells at the early stationary phase (10-days-old) was transferred into a 100 ml conical flask, and Bt_2cAMP or Ca^{2+}-ionophore A23187 in 100 μl of Na-citrate buffer (10 mM, pH 5.0) was added to the cell suspension at the final concns of 100 μM and 1 μM, respectively. The control culture received only the citrate buffer instead of the chemicals. In some experiments, carrot cultures were pre-incubated with 500 μM of verapamil or 4 μM of cycloheximide for 5 min prior to the addition of the inducers. They were incubated for 13 min at 26°, and cells and medium were separated by vacuum filtration. The cells were resuspended in 10 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 20 mM $MgCl_2$, and were homogenized by sonication. The homogenates were centrifuged at 10000 g for 15 min to remove cell debris. Resultant supernatants were dialysed against the homogenization buffer containing 2 mM EGTA to remove CAM which might bind PDE isoenzyme. EGTA was then removed by dialysis against the buffer without EGTA. Protein concns of the samples were determined by the method of ref. [18], and they served as the enzyme preparation of PDEs. The assay mixture consisted of 32–60 μg enzyme proteins, 20 mM Tris-HCl (pH 7.4), 8 mM $MgCl_2$, 0.02 mM [^{3}H]cAMP (7.4 kBq), and 2 mM EGTA or 0.1 mM $CaCl_2$ plus 10 μg of CAM (Ca^{2+}/CAM) in a total vol of 50 μl. The reaction was run at 37° for 1 hr, and stopped by boiling for 2 min. Aliquots (10 μl each) of the reaction mixture were applied onto a PEI-cellulose TLC plate (Merck), and the products were separated by the two-step development as described in Ref. [9]. As reported previously [9], the enzyme prepn included a potent phosphomonoesterase activity, and the main degradation product of cAMP was adenosine under the present experimental condition. Therefore, authentic adenosine and 5′-AMP were co-chromatographed in the TLC separation, and cellulose gels corresponding to the positions of these two compounds were scraped off. PDE activities in carrot cell extracts were estimated with the sum of the radioactivities of the two products. For the estimation of K_m and V values, the enzyme assay was carried out with 0.02–0.2 μM of cAMP in the presence of EGTA or Ca^{2+}/CAM. Values obtained in the presence of EGTA was subtracted from those of Ca^{2+}/CAM for the calculation of kinetic parameters of CAM-PDE.

Induction and assay of adenylate cyclase activity. Induction and assay of adenylate cyclase activity in cultured carrot cells were carried out essentially according to the method described in Ref. [9]. In brief, carrot cell culture (10 ml) was treated with 1 μM of forskolin, and after 5 min of the incubation, the cells were harvested by filtration. They were resuspended in 5 ml of 50 mM HEPES buffer containing 5 mM $MgCl_2$ and 0.5 M sucrose (pH 7.4), and were homogenized by sonication. Cell debris was removed by centrifugation (10000 g, 15 min). Resultant supernatants were dialysed against the HEPES–sucrose buffer, and employed as the enzyme prepn for the assay of adenylate cyclase. The assay mixture contained 45–83 μg enzyme proteins, 10 mM HEPES (pH 7.4), 0.1 M sucrose, 20 mM creatine phosphate, 40 μg of creatine kinase, 50 μg of myokinase, 5 mM 3-isobutyl-1-methylxanthine, 1 mM $MgCl_2$, 0.1–1 μM [^{3}H]ATP (7.4 kBq) and 2 mM EGTA in a total vol. of 100 μl. The mixtures were incubated at 37° for 1 hr, and the reaction was terminated by boiling for 2 min. The products were separated by TLC on a PEI-cellulose plate as described above, and radioactivities co-migrated with authentic cAMP were determined.

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