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Involvement of plasma membrane-located calmodulin in the response decay of cyclic nucleotide-gated cation channel of cultured carrot cells

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Abstract

Increase in cytoplasmic cyclic AMP concentration stimulates Ca^{2+} influx through the cyclic AMP-gated cation channel in the plasma membrane of cultured carrot cells. However, the Ca^{2+} current terminated after a few minutes even in the presence of high concentrations of cyclic AMP indicating that hydrolysis of the nucleotide is not responsible for stop of the Ca^{2+} influx. Cyclic AMP evoked discharge of Ca^{2+} from inside-out sealed vesicles of carrot plasma membrane, and it was strongly inhibited when the suspension of the vesicles was supplemented with 1 μM of free Ca^{2+}, while Ca^{2+} lower than 0.1 μM did not affect the Ca^{2+}-release. The Ca^{2+} flux across plasma membrane was restored from this Ca^{2+}-induced inhibition by the addition of calmodulin inhibitors or anti-calmodulin. These results suggest that Ca^{2+} influx initiated by the increase in intracellular cAMP in cultured carrot cells is terminated when the cytosolic Ca^{2+} concentration reaches the excitatory level in the cells, and calmodulin located in the plasma membrane plays an important role in the response decay of the cyclic nucleotide-gated Ca^{2+} channel.

Key words: Calmodulin; Cyclic AMP-gated cation channel; Plasma membrane; Response-decay; Signal transduction; *Daucus carota* L.

1. Introduction

The physiological role of cyclic AMP (cAMP) in higher plants has not been well established [1,2]. However, we have recently demonstrated [3] that one of the signal transduction mechanisms of the nucleotide in cultured carrot cells is a unique signal-crosstalking with Ca^{2+}-cascade. The content of cAMP is transiently elevated when the cells are appropriately stimulated, and its increase results in the activation of Ca^{2+}-channel on the plasma membrane without accompanying cAMP-dependent protein phosphorylation. Thereafter, the cytoplasmic Ca^{2+} concentration reaches a sufficient level to activate several Ca^{2+}- or Ca^{2+}/calmodulin-dependent processes. A similar mechanism of transmembrane signalling was reported in odor-sensitive animal cells [4–6], and it was also shown that the termination of cAMP-elicited Ca^{2+} influx is caused by the hydrolysis of cAMP in these cells [7]. Unlike in olfactory cells, however, in cultured carrot cells [3], cytoplasmic Ca^{2+} elevated by the stimulation of cAMP began to decrease even though the level of intracellular cAMP was significantly high. In addition, when Ca^{2+} influx was elicited by the treatment of the cells with dibutyryl cAMP, the cytoplasmic concentration of Ca^{2+} returned to the basal level after 12–18 min at which time the cAMP analog was still present in high concentrations [3]. These results indicate that the degradation of cAMP is not the direct reason for the response decay of the cAMP-gated cation channel of carrot cells. In the present experiments, we have attempted to elucidate the cellular component(s) which could be involved in the termination of Ca^{2+} influx elicited by cAMP. Several lines of evidence suggest that calmodulin located in the plasma membrane of carrot cells is an essential structure to terminate the inward current of Ca^{2+}, and the results obtained here propose a possible role of plasma membrane-located calmodulin of higher plant cells for which functions have not yet been determined.

2. Materials and methods

2.1. Chemicals

Calmodulin (bovine brain), trifluoperazine, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) were purchased from Wako Pure Chemicals (Osaka, Japan). Anti-bovine brain caldomulin (sheep, affinity purified IgG fraction) was obtained from Polysciences (PA, USA) and anti-sheep IgG (rabbit, conjugated with alkaline phosphatase) was from Cosmo Bio (Tokyo, Japan). $^{45}CaCl_2$ (specific activity 111.0 GBq/mmol) was obtained from New England Nuclear. All other chemicals were reagent grade.

2.2. Preparation of sealed plasma membrane vesicles from cultured carrot cells

Cultured carrot cells were grown in Murashige and Skoog's synthetic medium [8] on a reciprocal shaker [9], and plasma membrane rich fraction was prepared by the aqueous two-phase partitioning method

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[10] as described previously in detail [3]. In brief, cells in early stationary phase (10 days old) were harvested by filtration, and homogenized by sonic oscillation. The homogenates were centrifuged at 10,000 × g for 20 min, and the resultant supernatants were further centrifuged at 100,000 × g for 1 h. Precipitated microsomes were subjected to two-phase partitioning with 10 mM HEPES-NaOH buffer (pH 7.2) containing 250 mM sucrose, 30 mM NaCl, 5.6% (w/w) polyethylene glycol 3350 (Sigma) and 5.6% (w/w) Dextran T-500 (Pharmacia). The resultant upper phase was removed and mixed again with freshly prepared lower phase, and plasma membranes in the upper phase were recovered by centrifugation (156,000 × g, 30 min). The purity of the plasma membrane preparation thus obtained was accessed by measuring the activities of several marker enzymes, and the results have been reported previously [3].

2.3. Loading and discharge of $^{45}Ca^{2+}$

$^{45}Ca^{2+}$ was loaded into the inside-out vesicles of plasma membrane by the action of Ca^{2+}-ATPase according to the method of Graef and Weiler [11] with some modifications [3]. Plasma membrane preparation (600 μg protein as determined by the method of Bradford [12]) suspended in 25 mM HEPES-NaOH buffer (pH 7.2, containing 250 mM sucrose) was incubated with 1 mM ATP, 1 mM $MgCl_2$ and 1.3 μM $^{45}CaCl_2$ (74 kBq) in a total volume of 4 ml at 26°C for 1 h. The reaction was stopped by the addition of 6 ml of 1 mM EGTA in the HEPES-sucrose buffer, and the vesicles were recovered by centrifugation (156,000 × g, 30 min). They were successively washed with the HEPES-sucrose buffer containing 1 mM EGTA (twice), then with the same buffer without EGTA. Precipitated vesicles were resuspended in the HEPES-sucrose buffer, and an aliquot was removed to determine the total incorporation of $^{45}Ca^{2+}$ after the destruction of the vesicles with 1 ml of 5% (w/v) SDS. Ca^{2+}-loaded vesicles (15 μg protein in a 170 μl aliquot containing 0.37–0.52 kBq of $^{45}Ca^{2+}$) were firstly treated with EGTA (final concentration 1 mM), various concentrations of Ca^{2+} or 10 μM Ca^{2+} plus 5 μg calmodulin. These chemicals were dissolved in the HEPES-sucrose buffer, and the 20 μl aliquots were added to the suspensions of vesicles. In some experiments, appropriate amounts of $CaCl_2$ were added to the buffer containing 0.2 mM EGTA in order to adjust free Ca^{2+} concentrations to 0.1 and 1 μM assuming that the binding constant of EGTA-Ca^{2+} is 7.61×10^6 [3,13]. The mixtures were incubated at 26°C for 5 min, and then, cAMP (10 μl to give the final concentration of 10 μM) was added to stimulate $^{45}Ca^{2+}$-release from the vesicles. After a 10-min incubation, 1 ml of the HEPES-sucrose buffer was added as a carrier solution, and the mixtures were filtered through membrane filters (0.2 μM, Toyo Roshi). The filtrates containing the discharged $^{45}Ca^{2+}$ were mixed with a commercial scintillation cocktail (Amersham, ACS II), and their radioactivities were determined. When the $^{45}Ca^{2+}$-loaded vesicles were preincubated with 1 mM EGTA, 48–65% of $^{45}Ca^{2+}$ were released from the vesicles under the present experimental condition. Control treatment received the HEPES-sucrose buffer instead of cAMP.

2.4. Immunoblot analysis of calmodulin in the plasma membrane

Plasma membrane preparation obtained by two-phase method (20 μg protein each) was denatured by boiling in the presence of 2 mM EGTA or 20 μM $CaCl_2$, and the samples were subjected to SDS-PAGE (15% gel). Separated proteins were transferred to a nitrocellulose membrane (0.2 μm) in a semi-dry transfer cell (Bio-Rad, Transblot SD) as described previously [14]. After blocking with 1% (w/v) bovine serum albumin in 10 mM Tris-HCl-buffered saline (pH 7.4), the membrane was incubated with anti-calmodulin (1 μg/ml, diluted with the buffered saline containing 0.05% Tween 20) for 1 h. Then, it was washed and further incubated with anti-sheep IgG (conjugated with alkaline-phosphatase) for 30 min. Calmodulin was monitored by immersing the membrane into 100 mM Tris-HCl (pH 9.5, containing 100 mM NaCl and 5 mM $MgCl_2$) containing 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazorium chloride as the substrates.

3. Results and discussion

It is well known that plasma membranes of higher plant cells prepared by the aqueous two-phase partitioning method are composed of differently oriented sealed vesicles. Graef and Weiler [11] reported that incubation of the mixture of plasma membrane vesicles with Mg-ATP and $^{45}CaCl_2$ resulted in the selective loading of radiolabeled Ca^{2+} into the inside-out sealed vesicles by the action of Ca^{2+}-ATPase of the membrane which functions as a Ca^{2+}-pump to keep the intracellular level of Ca^{2+} low in the resting state, in vivo. It has been shown [3,15] that this Ca^{2+}-loaded inside-out vesicle is a suitable model to examine the Ca^{2+} influx across plasma membrane of plant cells since various intracellular conditions can be mimicked with the use of appropriate buffers suspending the vesicles.

In mammalian cells, Ca^{2+}-induced inactivation of Ca^{2+} channels is known as one of the common mechanisms of the channel regulation [16]. Therefore, we firstly examined the possible effect of Ca^{2+} on the discharge of the ions from the vesicles. $^{45}Ca^{2+}$-loaded vesicles were preincubated with EGTA or various concentrations of Ca^{2+}, and the discharge of $^{45}Ca^{2+}$ was initiated by the addition of cAMP. As shown in Table 1, cAMP-induced $^{45}Ca^{2+}$ flux from the plasma membrane vesicles was markedly inhibited in the presence of Ca^{2+} above 1 μM which almost corresponds to Ca^{2+} level of the cells in the excitatory state [3]. In contrast, Ca^{2+} below 0.1 μM, a similar level to that in the resting state [3], did not show any significant effect on the cAMP-stimulated Ca^{2+} flux. Pretreatment of the vesicles with 10 μM Ca^{2+} plus 5 μg of bovine brain calmodulin did not show any stimulatory or inhibitory effect. Therefore, it appeared that the exogenous calmodulin is not an important component to inhibit the discharge of the ions. These results suggested that the cellular components which are essential for the termination of cAMP-induced Ca^{2+} influx in cultured carrot are endogenously included in the plasma membrane of the cells, and elevation of cytoplasmic Ca^{2+} elicits the action of the machinery to close the channel.

Table 1
Effect of Ca^{2+} on the discharge of $^{45}Ca^{2+}$ from inside-out sealed vesicles of carrot plasma membrane

Preincubation	Discharge of $^{45}Ca^{2+}$ (%)	
	Experiment 1	Experiment 2
EGTA (1 mM)	100	100
Ca^{2+} (0.1 μM)	95	94
Ca^{2+} (1 μM)	2	2
Ca^{2+} (10 μM)	2	6
Ca^{2+} (10 μM) plus bovine brain calmodulin (5 μg)	4	7

$^{45}Ca^{2+}$-loaded vesicles were preincubated with EGTA, various concentrations of Ca^{2+} or Ca^{2+} plus calmodulin, and discharge of $^{45}Ca^{2+}$ was evoked by the addition of cAMP. In order to adjust the free Ca^{2+} concentrations to 0.1 and 1 μM, EGTA-Ca buffer was employed according to the method of Hetherrington and Trewavas [13]. Radioactivities released from the vesicles preincubated with EGTA were referred as 100% (0.24 and 0.31 kBq), and the results obtained from two independent experiments are shown as percentages.

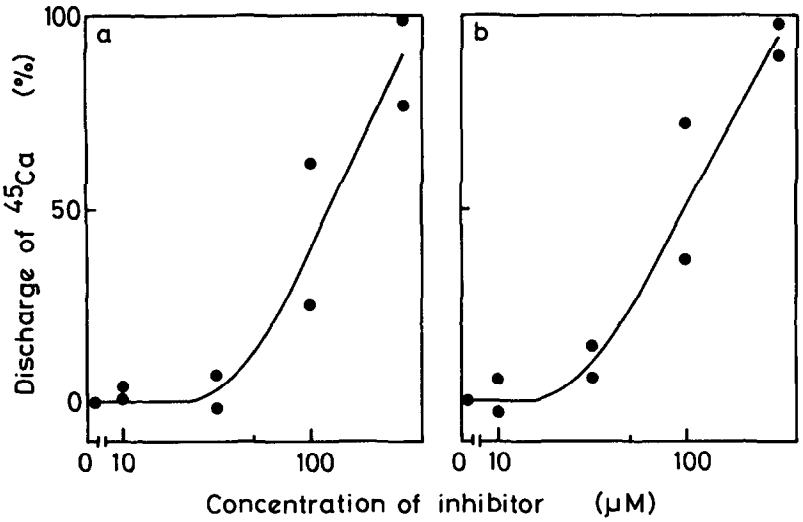

Fig. 1. Effect of calmodulin inhibitors on Ca^{2+}-induced inhibition of $^{45}Ca^{2+}$ discharge from plasma membrane vesicles evoked by cAMP. $^{45}Ca^{2+}$-loaded vesicles were preincubated with various concentrations of trifluoperazine (a) or W-7 (b) in the presence of 10 μM of Ca^{2+}, and the discharge of $^{45}Ca^{2+}$ was initiated by the addition of cAMP. Radioactivity released from the vesicles preincubated with EGTA was referred as 100% while that from 10 μM Ca^{2+}-treated vesicles without the calmodulin inhibitors was 0%. Results obtained from two independent experiments are shown as percentages.

Recently, Collinge and Trewavas [17] reported Ca^{2+}-dependent association of calmodulin with several proteins in the plasma membrane of pea. They also showed [17] that significant amounts of calmodulin are included in the lipid bilayer of pea plasma membrane (0.5–1.0% of total protein), and they supposed that this membrane-bound and located calmodulin would be involved in the signal transduction mechanisms although no direct evidence was available. In the next experiment, the possibility was tested whether or not the membrane-associated calmodulin is involved in the response decay of the cAMP-gated Ca^{2+}-channels of carrot cells. As shown in Fig. 1, both trifluoperazine and W-7, different classes of calmodulin inhibitors, were able to recover the $^{45}Ca^{2+}$-release from Ca^{2+}-induced inhibition in a dose-dependent manner. Fifty percent recovery of the $^{45}Ca^{2+}$-release was achieved with approximately 100 μM of the reagents, and these figures are comparable with or slightly higher than those reported for other calmodulin-dependent processes in plant cells such as activation of protein kinase [13], Ca^{2+}-ATPase [18] or induction of defense enzymes [19]. These reagents up to 500 μM did not show any significant effect on $^{45}Ca^{2+}$ release from the vesicles when the vesicles were preincubated with 2 mM EGTA, instead of 10 μM Ca^{2+} (data not shown). Therefore, it is likely that the recovery of $^{45}Ca^{2+}$-release from Ca^{2+}-induced inhbition by trifluoperazine or W-7 is due to their inhibitory activity against calmodulin, and therefore, this fact suggests that endogenous calmodulin included in the plasma membrane preparation would be an essential component for the termination of the action of cAMP-stimulated cation channel.

To confirm the hypothesis described above, we tested the effect of anti-calmodulin on the Ca^{2+}-inhibited discharge of $^{45}Ca^{2+}$ from the membrane vesicles of cultured carrot cells. In immunoblot analysis, only one protein in the plasma membrane preparation was found to react with the commercial anti-calmodulin raised against bovine brain, and showed the signal at the position corresponding to authentic calmodulin (Fig. 2). A migration shift in SDS-PAGE in the presence of EGTA or Ca^{2+} also supported the view that the antibody recognized only calmodulin among the proteins included in carrot plasma membrane. As shown in Fig. 3, this antibody significantly recovered the $^{45}Ca^{2+}$-release from the inside-out membrane vesicles in a dose-dependent manner from the Ca^{2+}-induced inhibition. The discharge of $^{45}Ca^{2+}$ recovered to 50% of the level with approximately 1 μg/ml IgG in the incubation mixture. In contrast, non-immune

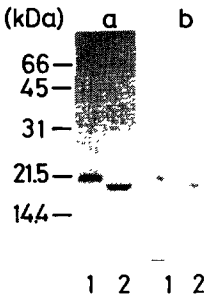

Fig. 2. Immunoblot analysis of calmodulin involved in the plasma membrane fraction of cultured carrot cells. One μg of bovine brain calmodulin (a) and 20 μg proteins of plasma membrane fraction of cultured carrot cells (b) were subjected to SDS-PAGE in the presence of 1 mM EGTA (1) or 20 μM $CaCl_2$ (2), and the proteins were stained with Coomassie blue (a) or probed by anti-calmodulin (b). Positions of molecular weight markers are shown as bars with numbers (kDa).

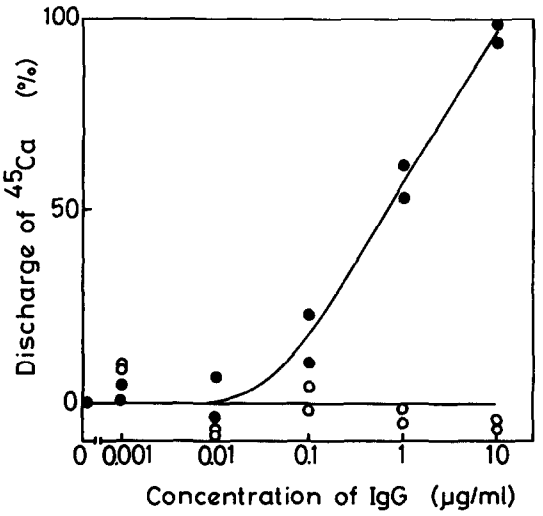

Fig. 3. Effect of anti-calmodulin on Ca^{2+}-induced inhibition of $^{45}Ca^{2+}$ discharge from membrane vesicles. $^{45}Ca^{2+}$-loaded vesicles were preincubated with various concentrations of anti-calmodulin IgG (●) or non-immune IgG (○) in the presence of 10 μM of Ca^{2+}, and the discharge was evoked by cAMP. Results obtained from two independent experiments are shown as percentages as described in Fig. 1.

IgG did not show any recovering activity to the Ca^{2+}-induced depression of the ion current, as far as tested. Inhibitory activity of this antibody against NAD kinase in carrot cell extract, a well known calmodulin-dependent enzyme in plants, was also tested according to the method of Refeno et al. [20], and it was found that 50% inhibition of the enzyme reaction was achieved with about 0.2 μg/ml of this anti-calmodulin (data not shown). Apparent different activities of the anti-calmodulin to the two calmodulin-dependent processes might be caused by the difference of the reaction systems. It is possible that calmodulin molecules associated with the membrane less efficiently contact with the antibody than the solubilized form. These observations strongly suggest that calmodulin in the plasma membrane of cultured carrot plays an important role in the response decay of the cyclic nucleotide-gated cation channels of the cells. Although Ca^{2+}-dependent calmodulin binding to several target proteins in the plasma membrane has been reported in pea [17], it seems that this is not the case in closing the cAMP-sensitive cation channel of carrot cells. Since $^{45}Ca^{2+}$-loaded vesicles of plasma membrane were repeatedly washed with EGTA-containing buffer to terminate the incorporation reaction of the radiolabeled ions, calmodulin bound to the membrane in Ca^{2+}-dependent manner, if any, should be removed during this process. Therefore, calmodulin involved in this transmembrane signaling process should be EGTA-stable, probably partially embedded form in the lipid bilayer as reported in pea [17].

We reported previously [21] that activities of adenylyl cyclase and phosphodiesterase of cultured carrot cells are strictly controlled by the intracellular Ca^{2+} concentration. It was shown that both synthesis and degradation of cAMP are switched on/off according to the change in cytosolic Ca^{2+} level. Results obtained here suggest that the Ca^{2+} entry into carrot cells triggered by cAMP is terminated by elevation of cytoplasmic Ca^{2+} followed by the activation of plasma membrane-located calmodulin, and therefore, Ca^{2+} introduced into carrot cytoplasm works not only in the activation of the Ca^{2+}-cascade but also in the termination of the excitatory state of the cells by closing the channel structure and, in parallel or as a later event, by degradation of the primary stimulant, cAMP.

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