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STIMULATION OF Ca^{2+}-PUMPING ATPase ACTIVITY IN CARROT PLASMA MEMBRANE BY CALMODULIN

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Key Word Index—*Daucus carota*; Umbelliferae; Ca^{2+}-pumping ATPase; plasma membrane; calmodulin; kinetic analysis; stoichiometry of enzyme reaction.

Abstract—Activity of Ca^{2+}-pumping ATPase located at the plasma membrane of cultured carrot cells was appreciably stimulated when the enzyme was associated with calmodulin (CAM). Affinity of the ATPase toward the substrates was increased by the addition of CAM, and K_ms of the enzyme for Ca^{2+} were estimated to be 11.4 and 0.7 μM in the absence and presence of CAM, respectively, while the values for ATP decreased only slightly (914 and 670 μM). In contrast, relative V values of the enzyme were essentially not changed even after the addition of CAM. This ATPase was capable of utilizing several ATP analogues, such as GTP, ITP, UTP and CTP, instead of ATP, and Ca^{2+}-transport driven by these alternative substrates was also stimulated by CAM. The molar ratio of Ca^{2+}-transport and ITP hydrolysis of the enzyme reaction was estimated to be 2:1. These observations suggested that CAM-induced stimulation of Ca^{2+}-ATPase activity at the plasma membrane of cultured carrot cells is mainly due to the increase in the affinity of the enzyme toward Ca^{2+} by association with the modulator protein, and the hydrolysis of 1 mol of ATP by the enzyme action results in the transport of 2 mol of Ca^{2+} across the membrane.

INTRODUCTION

It is well known that Ca^{2+} is an important second messenger in signal transduction mechanisms in higher plant cells as well as in other eukaryotic cells [1], and maintenance of low Ca^{2+} concentration in cytoplasm is an essential requirement for the non-stimulated cells. Cytoplasmic Ca^{2+} concentration of plant cells at the resting state is generally maintained at about 0.1 μM by the action of Ca^{2+}-transporting systems [2] which sequester the ion into internal organelles including endoplasmic reticulum, mitochondria and vacuoles, or mediate its efflux to cell exterior. It has been demonstrated that Ca^{2+}-pumping ATPase located at the plasma membrane plays a central role in Ca^{2+} efflux to apoplastic space [2]. Several characteristics of the Ca^{2+}-translocating ATPase have been reported from a wide range of plants [2]. However, some of the results are highly variable depending on the plant species. One of the most serious controversies on the properties of the ATPase is a role of calmodulin (CAM) in the regulation of the enzyme, and inconsistent observations on the CAM-dependency of the enzyme activity have been reported from several plants [3–6]. It is not clear, at present, whether this apparent discrepancy depends on the inherent nature of each plant or merely the difference of experimental methods. I assumed that the inconsistency about the role of CAM in controlling Ca^{2+}-ATPase activity resulted from, at least partly, use of the plasma membrane preparations containing considerable amounts of membranes of other organelles as the experimental materials. Recently, we have prepared a highly purified plasma membrane fraction of cultured carrot cells by aqueous two phase partitioning method [7], and reported that Ca^{2+}-pumping ATPase activity appreciably decreased by the treatment of the membrane with EGTA, CAM antagonists or anti-CAM IgG [7]. The ATPase activity of the EGTA-treated membrane restored to almost the control level by the addition of exogenous CAM [7]. These observations suggested that the activity of Ca^{2+}-translocating ATPase at carrot plasma membrane is stimulated by CAM, and this modulator protein associates with the enzyme in a Ca^{2+}-dependent manner and is readily removed by the treatment with the chelating reagent.

In the present experiments, I attempted to elucidate the biochemical mechanism by which Ca^{2+}-ATPase activity located at the plasma membrane of cultured carrot cells is enhanced by association with CAM. Kinetic parameters of the Ca^{2+}-transport reaction of the EGTA-treated plasma membrane were determined in the absence and presence of exogenous

Table 1. Summary of kinetic parameters of Ca^{2+}-ATPase located at the plasma membrane of cultured carrot cells in the absence and presence of CAM

	−CAM	+CAM
K_m for Ca^{2+} (μM)	11.4±2.8	0.7±0.3
K_m for ATP (μM)	914±84	670±57
Relative V	1.2±0.3	1.0

Results were expressed as the means and s.d. obtained from three independent experiments. V was expressed as the relative ratio to the velocities obtained for the ATPase in the presence of CAM as 1.0 in each set of the experiments.

CAM. Molecular stoichiometry of Ca^{2+}-transport and ATP hydrolysis of the ATPase-catalysing reaction was also examined employing ITP as the alternative substrate.

RESULTS AND DISCUSSION

Kinetic analysis of Ca^{2+}-pumping ATPase activity in the absence and presence of CAM

In order to elucidate the biochemical bases of the stimulation of Ca^{2+}-ATPase activity of cultured carrot cells by association with CAM, the kinetic parameters of Ca^{2+}-translocating reaction of the enzyme were determined in the absence and presence of exogenous CAM employing EGTA-treated plasma membrane. Ca^{2+}-pumping activities were determined by measuring incorporation of ^{45}Ca into sealed vesicles of plasma membrane, and the experimental data were analysed by double reciprocal plots. Primary reciprocal plots were determined at a series of concentrations of Ca^{2+} (0.15–2 μM) in the presence of ATP (2 mM), and similar experiments were carried out with a fixed concentration of Ca^{2+} (2 μM) and various concentrations of ATP (100–500 μM). Affinity of Ca^{2+}-ATPase for Ca^{2+} was appreciably increased by the association with CAM, and K_m values were decreased from 11.4 to 0.7 μM (Table 1). These figures are almost comparable to those of CAM-dependent Ca^{2+}-ATPase at the plasma membrane of animal cells [8]. As well as Ca^{2+}, affinity of the enzyme for ATP was also increased in the presence of CAM. However, the level of the increase was rather low as compared with Ca^{2+} (K_m; 914 and 670 μM in the absence and presence of CAM, respectively). An attempt to determine the velocities of the Ca^{2+} transport reactions in the absence and presence of CAM was unsuccessful since, in repeated experiments, V values differed. Probably a component of each batch of the membrane preparation was different and/or a part of the ATPase activity was lost during the purification processes. However, I found that the relative ratio of the V values of the Ca^{2+}-pumping reactions run in the absence and presence of CAM were almost identical throughout the experimental periods, therefore, the results were expressed as the ratio of the velocities of these two reactions. As shown in Table 1, unlike the affinities for the substrates, relative V values of the ATPase were found to be almost similar or slightly decreased by the addition of CAM. These results strongly suggested that, upon the binding of CAM, affinity of the carrot Ca^{2+}-ATPase toward Ca^{2+} is markedly increased, and this is the most important biochemical change responsible for the CAM-induced increase in the pumping activity of the enzyme.

It has been widely accepted that cytoplasmic Ca^{2+} level in higher plant cells at the resting state is about 0.1 μM, and it elevates to about 0.6–0.8 μM when the cells are appropriately stimulated [1, 2]. In the excited plant cells of high Ca^{2+} level, CAM is activated by binding to the ion, and is able to associate with various CAM-dependent proteins [1]. In contrast, Ca^{2+}concentration in the resting plant cells is too low to activate CAM, and this results in the dissociation of the modulator from its target proteins including Ca^{2+}-ATPase [7]. K_{ca} of the ATPase associated with CAM is similar to that of the cytoplasmic Ca^{2+} level of excited plant cells, while although the cytoplasmic Ca^{2+} concentration in the resting cells is quite low, K_{ca} of the ATPase without CAM markedly increased (Table 1). These observations apparently suggest that Ca^{2+}-ATPase at carrot plasma membrane plays an important role only in the excited cells as an 'acute' enzyme. However, Rasi-Caldogno *et al.* [6] pointed out that K_{ca} of about 10 μM decreased to about 0.1 μM if only the level of free Ca^{2+} was considered. This low K_m value of CAM-depleted Ca^{2+}-ATPase for Ca^{2+} is consistent with the transport protein involved in maintaining cytoplasmic Ca^{2+} concentration at the submicromolar range as a 'housekeeping' enzyme in the resting cells.

Ca^{2+}-translocating activity of the ATPase with alternative substrates

We have previously reported that Ca^{2+}-transport catalysed by plasma membrane-located ATPase of cultured carrot cells was also observed when ATP was replaced by GTP [7] as well as the ATPases from some other plant sources [9, 10]. In the present study, I tested the possibilities that ATP analogues other than GTP, also function in the Ca^{2+}-transport mediated by the enzyme, and the analogue-driven Ca^{2+}-transport is stimulated in the presence of CAM as well as in ATP. As shown in Fig. 1, all of the ATP-analogues, as far as tested, were capable of driving the Ca^{2+}-translocation, although the pumping efficiencies were somewhat lower than that of ATP. It was also demonstrated that these ATP analogue-driven Ca^{2+}-translocation reactions were appreciably enhanced in the presence of CAM.

In the following experiments, I attempted to determine the chemical stoichiometry of Ca^{2+}-transport and ATP hydrolysis of the Ca^{2+}-ATPase catalysing reaction. It is well known that ATP is shared as the common substrate by several functional proteins at

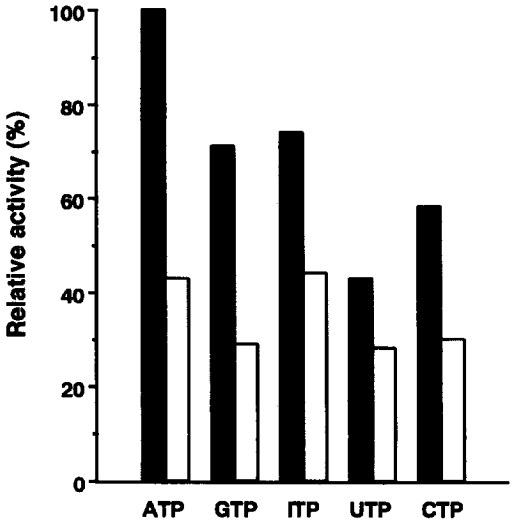

Fig. 1. Ca^{2+}-translocating activity of Ca^{2+}-ATPase at the plasma membrane of cultured carrot cells driven by ATP and its analogues in the presence and absence of CAM. ATP and its analogues (2 mM) were incubated with EGTA-treated plasma membrane of cultured carrot cells in the presence (closed column) or absence (open column) of CAM. Results were expressed as relative values in which ATP-driven Ca^{2+}-transport activity observed in the presence of CAM was taken as 100%.

plant plasma membrane including other classes at ATPases [2], and therefore measurement of Pi liberated from ATP does not reflect the hydrolysis of the substrate by the action of Ca^{2+}-ATPase. Therefore, an alternative substrate other than ATP should be employed for the above purpose, and, as shown in Fig. 1, ITP and GTP showed the potent activities in the ion transport among the ATP analogues. However, occurrence of GTP-binding protein at plant plasma membrane has recently been reported [11], implying the GTP is hydrolysed not only by Ca^{2+}-ATPase, but multiple functional proteins at the plasma membrane would also utilize this compound as the substrate. Therefore, I chose ITP as the alternative substrate, and hydrolysis of the compound by Ca^{2+}-ATPase was determined by measuring Pi released from the substrate. In a parallel experiment, ITP-driven Ca^{2+}-transport was also determined by the incorporation of ^{45}Ca into plasma membrane vesicles to compare the molar ratio of the two reactions. The ratio of Ca^{2+}-transport and ITP hydrolysis catalysed by the ATPase was estimated to be 1.8:1 and 1.9:1 in two independent experiments suggesting that hydrolysis of 1 mol of ATP by Ca^{2+}-ATPase results in the transport of 2 mol of Ca^{2+} to apoplastic space across the plasma membrane of cultured carrot cells. In animal cells, the ratio of Ca^{2+}-transport and ATP hydrolysis was shown to be 1:1 for plasma membrane Ca^{2+}-ATPase and 2:1 for endoplasmic reticulum-located enzyme, respectively [8]. The present finding, 2:1 ratio of Ca^{2+}-translocation versus ATP hydrolysis of plasma membrane-located Ca^{2+}-ATPase of cultured carrot cells, may be an important difference in the chemical stoichiometry of the ion transport by this class of enzyme between plant and animal cells.

EXPERIMENTAL

Chemicals. EGTA and CAM (bovine brain) were purchased form Wako Pure Chemicals while ATP, GTP, UTP, CTP and ITP were from Boehringer Mannheim. $^{45}CaCl_2$ (sp. act. 111 GBq $mmol^{-1}$) were obtained from New England Nuclear. All other chemicals were reagent grade.

Preparation of sealed plasma membrane vesicles of cultured carrot cells. Cultured carrot cells were grown in 70 ml of Murashige and Skoog's liquid medium [12] on an Innova 2300 rotary shaker (200 rpm) at 26° [7]. Plasma membrane fr. was prepd by aq. 2 phase partitioning method as described previously [7, 13]. In brief, the microsome fr. of 10-day-old cultured carrot cells was subjected in partitioning with 10 mM Hepes–NaOH buffer (pH 7.2) containing 250 mM sucrose, 30 mM NaCl, 5.6% polyethylene glycol 3350 (Sigma) and 5.6% Dextran T-500 (Pharmacia). The resultant upper phase was removed and mixed again with freshly prepd lower phase, and plasma membranes in the upper phase were recovered by centrifugation (156 000 *g*, 30 min). Purity of the membrane was assessed by measuring several marker enzymes and the results were reported previously [13]. Purified plasma membrane was washed with 25 mM Hepes–NaOH buffer (pH 7.2) containing 250 mM sucrose and 2 mM EGTA by stirring 1 hr at 4° to remove CAM associated with the membrane. EGTA-treated membrane was then washed with the Hepes–sucrose buffer without EGTA by centrifugation to remove the chelating reagent.

Assay of Ca^{2+}-ATPase activity. Ca^{2+}-translocating activity of Ca^{2+}-ATPase across the plasma membrane of cultured carrot cells was determined by the incorporation of ^{45}Ca into inside-out sealed vesicles of the membrane as described previously [7, 13] with some modifications. In the standard experiments, plasma membrane prepn (*ca* 80 μg protein $assay^{-1}$ as determined by the method of ref. [14]) suspended in 25 mM Hepes–sucrose buffer was incubated with 2 mM ATP, 1 mM $MgCl_2$ and 2 μM of $^{45}CaCl_2$ (74 kBq) in the absence or presence of 10 μg of CAM in a total vol of 500 μl. The mixture was incubated at 37° for 1 hr, and the reaction was terminated by the addition of 500 μl of the Hepes–sucrose buffer containing 2 mM EGTA. The mixt. was passed through a nitrocellulose membrane filter (Advantec, 0.22 μm), and the filter was washed twice with 2 ml of the Hepes–sucrose buffer containing 2 mM EGTA. The radioactivity remaining on the filters was determined. In some experiments, the ATPase activity was determined with ITP as the alternative substrate. ATP in the standard assay mixt. was replaced by 2 mM ITP, and ITP-hydrolytic activity of the enzyme was estimated by measuring Pi liberated from the substrate according to the method of ref. [15].

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