



PROTON PUMP ACTIVATION AND GROWTH PROMOTION BY CINCHOMERONIC ACID IN RADISH

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Abstract—Cinchomeronic acid (CA, 3,4-dicarboxypyridine or 4-carboxynicotinic acid) promoted hypocotyl elongation of radish seedlings when added to the medium or applied to the hypocotyls. On microscopic observation of epidermal cells of the plant, the length of each cell was elongated several times when compared to those of the controls. The mechanism of the plant-growth promotion by CA was investigated with plasma membrane vesicle prepared from radish seedlings. CA elevated the V_{max} of vanadate-sensitive H^+ -ATPase in plasma membrane vesicle by 1.8 times. It was found that CA and quinolinic acid were activators, and isocinchomeronic acid and 2,4-lutidinic acid were inhibitors for the H^+ -ATPase. These results reflected the effects of these compounds on plant growth. It is suggested that CA activates ATP-dependent proton transport and leads to plant-growth promotion by acid.

INTRODUCTION

We have studied the effects of niacin-related compounds on (i) various plants, animals and bacteria, and (ii) the metabolic pathways of niacin in relation to

growth, differentiation, etc. of these organisms [1–9]. It was found for the first time that cinchomeronic acid (CA) promoted the growth of duckweed [1], radish seedlings [4, 8], and *Escherichia coli* B [7]. CA absorbed by radish seedlings was found to be in the free form and was not metabolized into other compounds nor bound with high M_r compounds [4, 8]. Radish seedlings grown on a medium containing CA at 10 mM were about twice as tall after 10 days from sowing than those grown on control medium without CA [4, 8]. On microscopic examination of the epidermal cells of the hypocotyls, each cell in the seedlings grown on the CA-containing medium was elongated several times as compared to those of the control [4]. Furthermore, CA-related compounds, e.g. quinolinic acid (2,3-dicarboxypyridine, 2,3-DCP), also increased hypocotyl elongation, but isocinchomeronic acid (2,5-DCP) and 2,4-lutidinic acid (2,4-DCP) inhibited the elongation [8]. The structures of these compounds are shown in Fig. 1.

Two factors are known to contribute to the growth of plants. The first is an increase in the number of cells in limited parts in plant body, i.e. meristematic tissues at the end of the hypocotyl and root. The second is the enlargement in the size of each cell. Generally speaking, the cell volume in plants increases ca 10–20 times

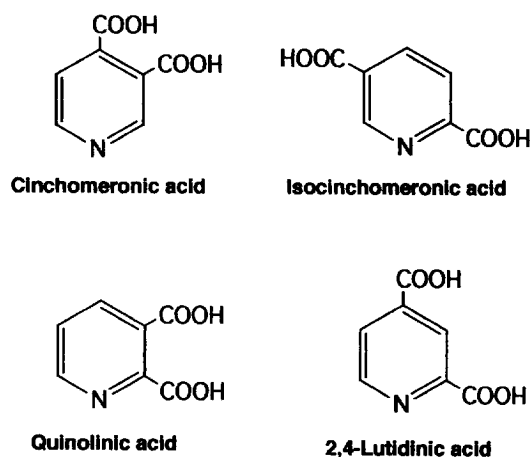


Fig. 1. Structures of cinchomeronic acid and its related compounds. Cinchomeronic acid and quinolinic acid increase hypocotyl elongation, but isocinchomeronic acid and 2,4-lutidinic acid inhibit elongation.

Table 1. Effect of cinchomeric acid on the growth of radish seedlings when applied on the hypocotyls

Measured part	Control	CA applied
Hypocotyl length (cm)	8.97 $\pm$ 0.219	14.05 $\pm$ 0.293
Hypocotyl diameter (mm)	1.33 $\pm$ 0.015	1.29 $\pm$ 0.028
Leaf area (cm ²)	0.38 $\pm$ 0.024	0.33 $\pm$ 0.018

Values shown here are mean $\pm$ SE from 10 samples.

Difference of mean between control and CA applied group is significant ($p < 0.001$) only in hypocotyl length by Student's *t*-test.

This kind of growth promotion is also caused by the plant hormone, auxin. Thus, permeability of the plasma membrane is enhanced and the proton pump is activated by the auxin [11–15]. The inner surface of the cell wall is acidified by the H⁺ formed and the bonds between the polysaccharide chains in the cell wall are weakened. The cell wall is then enlarged and loosened. As this occurs, water is incorporated into the cell and stored in vacuoles. Thus, the cell is enlarging due to the increasing size of the vacuole. Finally, elongation of the plant body is accelerated.

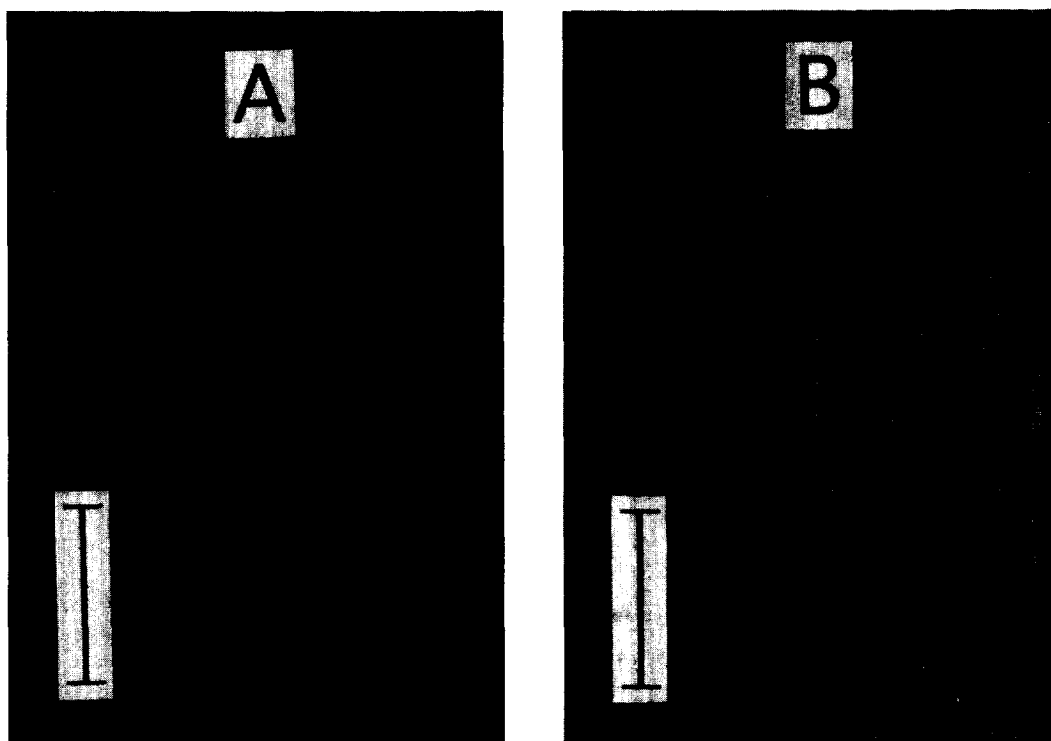
We have investigated whether or not plant-growth promotion by CA is due to a similar mechanism to that just described. The results show for the first time that CA activates the proton pump, H⁺-ATPase (EC 3.6.1.35), in the plasma membranes in radish seedlings.

This activation is thought to lead to promotion of plant growth.

RESULTS

Effects of application of CA to stems of radish seedlings on growth and the epidermal cells of the hypocotyls

As shown in Table 1, hypocotyl diameter and leaf area in radish seedlings treated with CA were the same as those without CA, but the hypocotyl length was *ca* 1.6 times longer in the seedlings with CA than those without CA. Microphotographs of the epidermal cells in hypocotyls, after staining with toluidine blue O, are shown in Fig. 2. Cell length was elongated several



times by CA. Similar results were obtained when CA was added to the medium instead of applying it directly to the hypocotyls [4].

Purity of prepared plasma membrane vesicles

The purity of prepared plasma membrane vesicles was investigated by using specific inhibitors for various ATPases in each subcellular fraction. Vanadate inhibited ATPase activity strongly, as shown in Table 2. Inhibition by other compounds was rather weak. These results mean that the contamination of membranes from vacuoles and mitochondria was small. The prepared plasma membrane vesicle showed the same inhibition profile as standard ATPase from porcine cerebral cortex (Sigma Chemical Co.). Hence, the purity of the prepared plasma membrane vesicle was high. Furthermore, Mg^{2+} was required for ATPase activity in the prepared plasma membrane vesicle. This preparation of plasma membrane vesicles was used in the following experiments.

K_m value for ATP in the ATPase reaction

The K_m value for ATP obtained from a Lineweaver-Burk plot of the data obtained on incubating the plasma membrane vesicles with various ATP concentrations was 0.59 mM.

Effect of CA on the ATPase activity

The ATPase activity was determined at various concentrations of CA in the reaction mixture. As shown in Fig. 3, CA activated the reaction and the most effective concentration tested was 10 mM, at which concentration a 29.6% activation was observed. The ATPase activity of the plasma membrane treated with 0.2% (w/v) deoxycholate showed an 88.5% activation, but this activation was not affected on addition of CA at 10 mM.

Effect of CA on the H^+ -ATPase activity

The H^+ -ATPase activity was determined at various concentrations of ATP in the presence or absence of 10 mM CA by fluorescence quenching of quinacrine.

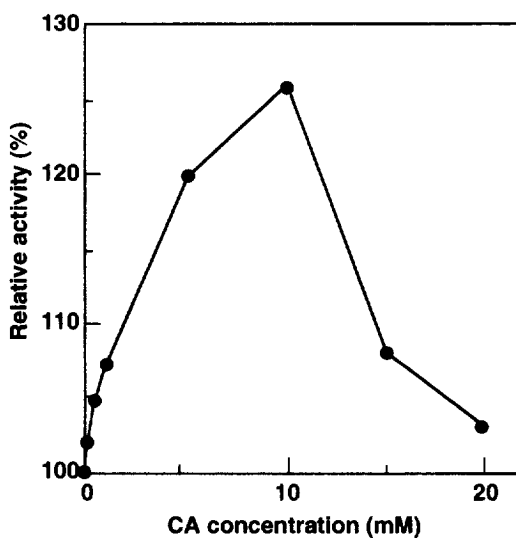


Fig. 3. Effect of cinchomeronic acid concentration on ATPase activity.

CA activated the H^+ -ATPase reaction in an [ATP]-dependent manner. Moreover, 0.1 mM vanadic acid inhibited the ATPase reaction in both the absence and presence of CA (10 mM). The inhibition rate with 0.5 mM ATP was 78.0%, as shown in Fig. 4. This concentration of vanadic acid was the same as that at which ATPase was inhibited.

Elevation of V_{max} in H^+ -ATPase reaction by CA

The V_{max} of the H^+ -ATPase reaction in the presence or absence of 10 mM CA in the reaction mixture was 27.8 and 15.2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively, when calculated by the Lineweaver-Burk plot. CA thus activated the H^+ -ATPase in the plasma membrane vesicles by raising the V_{max} 1.8-fold.

Effects of CA and related compounds on the H^+ -ATPase activity

Whether or not the activation of ATP-dependent H^+ -ATPase activity was specific to CA was investi-

Table 2. Effect of various inhibitors on the ATPase activity in the plasma membrane vesicle fraction prepared from radish seedlings

Inhibitor	Concentration (mM)	Inhibition (%)
NaN_3	1	17.6
Oligomycin	50 mg l^{-1}	18.7
KNO_3	50	16.9
Ammonium molybdate	0.1	24.8
Vanadate	0.1	78.0

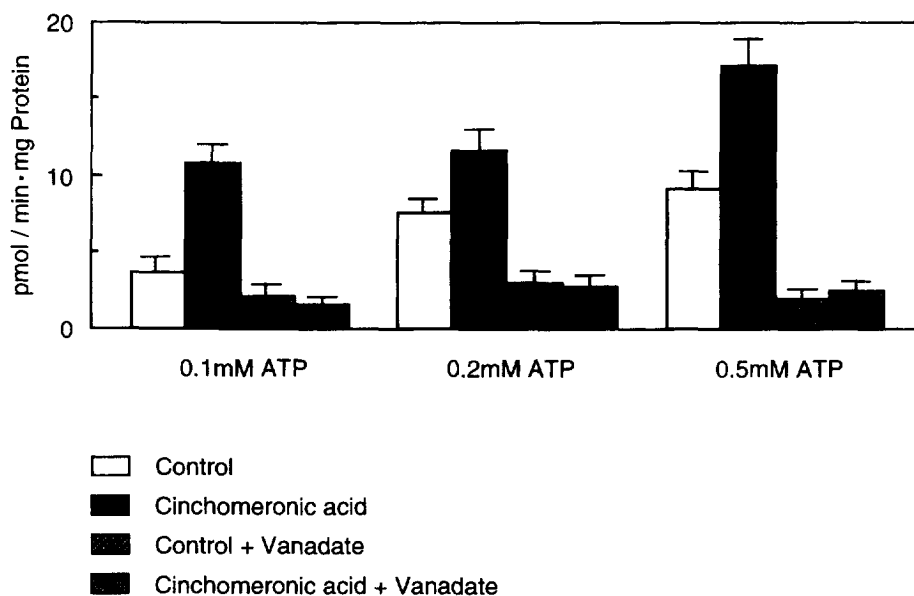


Fig. 4. Effect of cinchomeronic acid on vanadate-sensitive H^+ -ATPase activity. H^+ -ATPase activity in plasma membrane vesicle was determined by measuring fluorescence quenching of quinacrine, which was incorporated into the vesicles with the protons. Cinchomeronic acid and vanadate were added to each reaction mixture at 10 and 0.1 mM, respectively. Values shown here are mean $\pm$ SE from 5 experiments. Difference of mean between control and CA added groups is significant ($p < 0.001$) at 0.1, 0.2 and 0.5 mM of ATP by Student's *t*-test, respectively.

gated by using CA-related compounds, i.e. 2,3-DCP, dinicotinic acid (3,5-DCP), at 10 mM each in the reaction mixture. As shown in Fig. 5, CA was the most effective compound tested (47.4% activation). 2,3-DCP

also activated the enzyme reaction (32.0% activation) at 0.5 mM ATP. However, 2,5- and 2,4-DCP inhibited the reaction by 47.4 and 57.8% at 0.5 mM ATP, respectively.

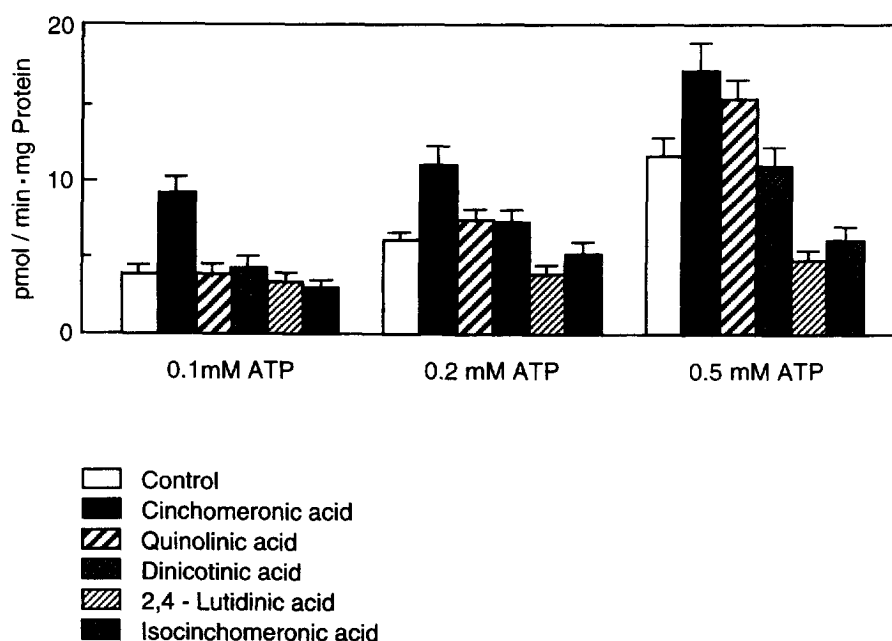


Fig. 5. Effects of cinchomeronic acid and its related compounds on H^+ -ATPase activity. The assay method was essentially the

DISCUSSION

As mentioned in the previous paper [4], the hypocotyl lengths of radish seedlings grown on a medium containing 10 mM CA were elongated about two-fold and the epidermal cells of the hypocotyls were elongated several times as compared to those of the controls. Similar results were also obtained when CA was applied to the hypocotyl in the present study. The growth promotion of radish seedlings by CA is thought to be explainable in terms of the theory proposed by Hayashi [16]. Namely, the growth promotion by CA may be due to the following successive phenomena: (1) enhancement of the permeability of the plasma membrane, (2) activation of the proton pump, and (3) loosening of the cell wall.

The purity of the prepared plasma membrane was investigated by using various inhibitors for each ATPase. Oligomycin and NaN_3 [17] are specific inhibitors of mitochondrial ATPase, while KNO_3 [17], ammonium molybdate [17] and vanadate [18] are specific inhibitors of the vacuole ATPase, acid phosphatase and plasma membrane ATPase, respectively. The inhibition by vanadate was the strongest and the inhibition rates of the other inhibitors were weak. Thus, the contamination with vacuole membranes and mitochondrial membranes was small, i.e. the preparation was derived almost entirely from the plasma membrane.

By using this preparation, the effect of CA on the ATPase in the plasma membrane of the vacuole was investigated. CA activated the enzyme activity and was most effective at 10 mM. At this concentration of CA, the highest plant-growth promotion was observed. It is suggested that there is a close relationship between growth promotion of radish seedlings and activation of ATPase activity. CA is not easily incorporated into plants and once taken up it remains in the free form and is not bound to high M_r compounds as shown in previous experiments with $[^{14}\text{C}]\text{CA}$ [4, 8]. Thus, it is thought that the high concentration of CA required for the activation of ATPase is due to the low permeability of the plasma membrane to CA. However, the plasma membrane after treatment with detergent, 0.2% (w/v) deoxycholate, showed a two-fold increase in ATPase activity, but CA did not affect it. It was reported that deoxycholate removed the protein kinase bound to ATPase [19]. This suggests that the mechanism of the activation of ATPase activity by CA is different to that for deoxycholate.

CA also activated the H^+ -ATPase. Moreover, the CA-activated H^+ -ATPase reaction was also inhibited by vanadate, and CA raised the V_{max} in the H^+ -ATPase reaction. Hence, the ATP-dependent proton pump in the plasma membrane is activated by CA. These results suggest that the growth promotion of radish seedlings by CA is due to growth promotion by acid [20]. Namely, raising the V_{max} enhances the proton flux from plant cells, and the inner surface of the

kind of proton flux has already been observed in corn roots following treatment with auxin, but the K_m value for ATP in the H^+ -ATPase reaction was decreased by ca 50% of its original value [21]. CA did not induce ATPase in radish seedlings, but activated it.

CA activated the proton pump in the plasma membrane mostly among tested DCP compounds as shown in Fig. 5 and 2,3-DCP also activated it, but 2,5-DCP and 2,4-DCP acid inhibited it. CA, 2,3-DCP and 3,5-DCP activated the growth of radish seedlings, and 2,5-DCP and 2,4-DCP inhibited the growth at 10 mM as already reported [8]. There is a good correlation between the activators and inhibitors of the proton pump and their effects on plant growth. Two carboxyl groups, one at C-3 of the pyridine ring and the other at C-2 or C-4, seem to be required for the activation of H^+ -ATPase activity.

CA activated the activity of ATPase by 29.6%, but it activated the activity of H^+ -ATPase by 47.4%. The reason for this difference in activation by CA may be due to the secondary proton flux of the H^+/K^+ -ATPase of the plasma membrane after activating the H^+ -ATPase. It has been reported that H^+/K^+ -ATPase exist widely in plant plasma membranes for K^+ transport at different locations from H^+ -ATPase [22–24]. The proton flux due to H^+ -ATPase may be activated by H^+/K^+ -ATPase. Thus, the total proton flux might be measurable by fluorescence quenching of quinacrine by H^+ -ATPase and H^+/K^+ -ATPase activities.

In summary, it is suggested that the plant-growth promotion by CA is acid growth due to the activation of the H^+ -ATPase in the plasma membrane as a result of raising its V_{max} . Many reports on plant-growth promotion by auxin and the mechanism of it have been published [11–15], but no reports of the effects of any analogues of vitamins like CA have been found.

EXPERIMENTAL

Plant material. Radish seeds (*Raphanus sativus* L. Shijunichi), purchased from Atariya Farm, Chiba, Japan, were selected for uniformity in size. Whole hypocotyls of the seedlings were collected after cultivation as described below and used for experiments.

Application of CA solution on the surface of radish hypocotyls and microscopic observation of the hypocotyl cells. Medium (3 ml) containing agar (pH 5.5 with KOH) in a test tube (18 × 180 mm) with an aluminium cap was autoclaved for 10 min at 120°. The seed, after sterilization with 70% EtOH, was then sown on to it. The test tubes containing seeds (one seed/test tube) were incubated in a mericlone rotary culturing instrument (Nippon Medical & Chemical Instruments, model LH-500RD) at 25° in the dark. Details of the medium and culture conditions were as described in a previous paper [4]. After a 5-day-incubation period,

H₂O was applied to the hypocotyl instead of CA soln. Hypocotyl length, hypocotyl diameter at the middle of the hypocotyls and leaf area were measured by the methods described already [4]. Average values obtained from 10 different seedlings are reported in this paper. Epidermal cells of the hypocotyls were observed with a microscope system (Olympus, model IMT-2-21 equipped with a camera, Olympus, model OM-4) at a magnification of $\times 20$ for both the eyepiece and objective lenses. Cell walls were stained with 0.1% toluidine blue O dissolved in 0.1 M K-Pi buffer, pH 7.0.

Preparation of plasma membrane vesicle. Prepn of plasma membrane vesicle from the hypocotyls of radish seedlings grown for 10 days on the medium without CA by the same methods as described above was carried out by means of an aq. polymer 2-phase system [25–29] under cooling at 0–4°.

Assay of ATPase. The reaction mixt. contained 50 mM Mes/Tris buffer (pH 6.5), 3 mM ATP, 3 mM MgSO₄, 50 mM KCl, 50 μ g membrane protein, and various inhibitors in a total vol. of 1 ml. Incubation was carried out at 30° for 30 min. Inorganic phosphate liberated from ATP by the enzymic reaction was determined by the molybdenum blue reaction [30]. To the reaction mixt., 0.2 ml 2.5% ammonium molybdate in 5 M H₂SO₄ (to stop the reaction and to develop the colour), 0.2 ml 1% NaHSO₃ (for stabilizing the colour), 20 μ l 5% SnCl₂ (as reducing agent) were added to a final vol. of 5 ml. After 15 min, the absorbance at 700 nm was measured. Phosphate concn was determined from a calibration curve prepd by using standard phosphate solns.

Assay of H⁺-ATPase. ATP-dependent transport of protons into the membrane vesicles, was determined by measuring the fluorescence quenching of quinacrine, which was incorporated into the vesicles with the protons. The reaction mixt. contained buffer soln [9.0% (w/w) sorbitol, 50 mM Tris, 20 mM MgSO₄, 100 mM KCl, pH 7.0 with Mes], 1 μ M quinacrine and 200 μ g membrane protein. The reaction was started by addition of ATP to give a final concn of 3 mM. The total vol. of the reaction mixt. was 2 ml. When the effects of CA and vanadate were investigated, these compounds were added to each reaction mixt. at 10 and 0.1 mM, respectively. Fluorescence was measured with a fluorescence spectrophotometer (Hitachi, model 650-60); excitation wavelength 423 nm; emission wavelength 502 nm; room temp.

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