J. Plant Biol. 40 (4) : 304-310 (1997)

Relationship Between IAA-induced Elongation Growth and Plasma Membrane NADH Oxidase in Soybean (*Glycine max* Merr.) Hypocotyls

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A relationship between the activity of NADH oxidase of the plasma membrane and the IAA-induced elongation growth of hypocotyl segments in etiolated soybean (*Glycine max* Merr.) seedlings was investigated. The plasma membrane NADH oxidase activity increased in parallel to IAA effect on elongation growth in hypocotyl segments. Actually, NADH oxidase activity was stimulated 3-fold by 1 μM IAA, and the elongation rate of segments was stimulated 10-fold by 10 μM IAA. The short-term elongation growth kinetics, however, showed that the IAA-induced elongation of hypocotyl segments was completely inhibited by plasma membrane redox inhibitors such as actinomycin D and adriamycin, at 80 μM and 50 μM respectively. In addition, 1 mM actinomycin D inhibited the IAA-stimulated NADH oxidase activity by about 80%. However, adriamycin had no effect on NADH oxidase activity of plasma membrane vesicles. Based on these results, the plasma membrane redox reactions seemed to be involved in IAA-induced elongation growth of hypocotyls, and the redox component responding to IAA was suggested to be NADH oxidase.

Keywords : NADH oxidase, elongation, IAA, adriamycin, plasma membrane

Since the discovery of redox activities associated with plasma membranes of eukaryotes, the correlation between growth-regulating hormones and redox activities has been investigated. Redox activities of isolated plasma membrane vesicles of liver and fat cells were responsive to insulin and glucagon (Goldenberg *et al.*, 1979). A natural electron donor and an acceptor for the hormone-responsive redox system of animal cells have been described as cytoplasmic NADH (Navas *et al.*, 1986) and external diferric transferrin (Crane *et al.*, 1985), respectively. Based on the fact that impermeable ferricyanide can stimulate growth of serum-deficient melano cells, it was considered that a redox function of the iron at the cell surface could be important to the control of cell growth (Ellem and Kay, 1983). Moreover, Ellem and Kay(1983) suggested that the identity of a hormone-responsive redox component would probably be an NADH-ferricyanide oxido-reductase which acts as a transmembrane electron carrier.

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In the case of plants, the situation is less clear. Growth of plant cells is inhibited rather than stimulated by external oxidants such as ferricyanide and ascorbate (Polevoy and Salamotova, 1977). Morré *et al.* (1988) also reported that a hormone-responsive redox component is an external NADH oxidase rather than an NADH-ferricyanide oxido-reductase. It has been suggested that the auxin-stimulated cell elongation accompanying acidification of cell walls in plants results from the activation of H^+-ATPase at the plasma membrane (Brummer and Parrish, 1983). However, Lin (1984) showed that the basal activity of oxygen consumption of the plasma membrane and its stimulation by external NADH are not retarded by application of H^+-ATPase inhibitors such as vanadate and diethylstibesterol. These results imply that the other system as well as an H^+-ATPase can establish a proton gradient across the plasma membrane. This implication, in fact, indicates an important role of the plasma membrane redox reactions associated with H^+ excretion (Morré, 1989). Although a key role of plasma membrane H^+-ATPase in auxin-induced growth stimulation is well known, the func-

tion of plasma membrane redox reactions could also be emphasized as a contributing factor for the proton gradient through regulating the NADH oxidase activity (Morré *et al.*, 1988).

Therefore, the present study was carried out to clarify the role of plasma membrane redox reactions in IAA-induced growth of hypocotyl segments from soybean seedlings. To do so, we first examined the IAA-induced short-term growth kinetics of hypocotyl segments, and then the existence of redox reactions was tested in the plasma membrane vesicles isolated from the same hypocotyl segment tissues by aqueous two-phase partitioning. Secondly, the activity of plasma membrane NADH oxidase in response to IAA was compared to the IAA-induced growth response in hypocotyl segments. In addition, the regulation of plasma membrane redox reactions by IAA was also investigated.

MATERIALS AND METHODS

Preparation of Plant Material

Soybean (*Glycine max* Merr.) seeds were surface-sterilized in 1% sodium hypochlorite solution for 30 min and then immersed for 20 h in distilled water with aeration. These seeds were sown and allowed to germinate on vermiculite moistened with distilled water in the dark at 27°C for 2 to 3 days. Abraded 1 cm or 2 cm segments of hypocotyls below the apical hook were excised and used for elongation and plasma membrane vesicle isolation, respectively. For removal of the epidermal cuticle, hypocotyls were abraded 10 times with fine sea-sand powder by pulling the hypocotyls between thumb and forefinger. The efficiency of cuticle removal and the degree of epidermal damage were confirmed with neutral red test and Evans blue test, respectively.

Measurement of Hypocotyl Growth

Growth kinetics was measured by one or two procedures. For measurement of long-term growth, ten segments were floated on 10 mL of medium in a glass tube. A stream of air was bubbled through the medium and the tubes were placed in a thermostated water bath (27±0.2°C). Segment elongation was read at suitable intervals on a millimeter scale. For measurement of short-term growth, a single segment was placed in a linear-displacement transducer (Serie 605-2.5, Erichsen, Wuppertal, FRG), which has a sensitivity of 1.68 mV/μM and permits the measurement of segment elongation rates with an accuracy of ±1 μM/min (Kutschera and Schopfer, 1985). The voltage output of the transducer was fed into a digital meter displaying both the actual voltage and the voltage change per unit time (set to 1 min). Transducer voltage output (i.e., segment extension rate) was recorded with a two-channel chart recorder set to 6 cm/h.

Isolation of Plasma Membrane Vesicles

Preparation of the plasma membrane-enriched vesicles from soybean hypocotyl segments followed the procedure of Larsson *et al.* (1987) with slight modification. Segments (70 g) were chopped with razor blades in 70 mL of homogenization buffer (50 mM Tris-Mes, pH 7.5, 0.3 M sucrose, 1 mM $MgCl_2$, 1 mM PMSF). The homogenate was filtered through one layer of Miracloth to remove debris and cell walls and centrifuged for 10 min at 6,000 *g*. The supernatant was recentrifuged at 60,000 *g* for 30 min and the pellets were resuspended in 0.25 M sucrose with 5 mM potassium phosphate (pH 6.8). Plasma membrane vesicles were prepared using a 16 *g* aqueous two-phase partitioning system. Resuspended 60,000 *g* pellets were mixed with 6.4% (w/w) polyethylene glycol 3350 (Sigma), 6.4% (W/W) Dextran T500 (Sigma), 0.25 M sucrose, and 5 mM potassium phosphate (pH 6.8). After mixing the tubes by 40 inversions the phases were separated by centrifugation at 750 *g* for 10 min. The lower phase was repartitioned with a fresh upper phase, and the two upper phases were repartitioned twice with fresh lower phases. The upper phases were diluted approximately two fold with buffer and collected by centrifugation at 100,000 *g* for 30 min. The resultant plasma membrane vesicles after centrifugation were resuspended in 1 mL of 5 mM potassium phosphate buffer (pH 6.8) containing 0.25 M sucrose and then protein content was determined by Bradford's (1976) method.

Measurement of Oxygen Consumption Activity of Plasma Membrane Vesicles

The oxygen consumption rate of isolated plasma membrane vesicles was assayed at 23°C using a Clark-type oxygen electrode (Bachofer, FRG) to characterize the property of the plasma membrane redox system (Møller and Bérezi, 1985). The basal activity of oxygen consumption was determined in 1 mL of 10 mM K-Mes (pH 6.0) buffer containing plasma membrane vesicles equivalent to 0.1 to 0.3 mg protein and 0.25 M sucrose. The kinetics of oxygen consumption

on the same plasma membrane vesicles was also assayed in the same medium after addition of 1 mM NADH, 2 mM SHAM, 2 mM KCN, 16 units catalase and 10 μM antimycin A.

Assay of NADH Oxidase Activity

NADH oxidase activity of purified plasma membrane vesicles was assayed by the quantitative estimation of NADH in a reaction medium using an enzyme cycling method (Matsmura and Miyachi, 1980). The 1 mL of reaction medium was composed of 10 to 20 μg of membrane vesicles, 25 mM Tris-HCl (pH 7.0), 0.1 M sucrose, 10 mM $CaCl_2$, 10 mM KCl and 10 mM NaCl. The enzyme reaction was initiated by adding 10 μM NADH. After 10 min incubation at 25°C, the reaction was stopped by adding 1 mL of 0.1 N NaOH and then a 200 μl aliquot of this medium was subjected to enzyme cycling in order to determine the decrease of NADH.

RESULTS AND DISCUSSION

Elongation Growth of Hypocotyl Segments by IAA

The dose-response curves for IAA in regulating elongation of 1-cm segments of the soybean hypocotyls is illustrated in Fig. 1. The elongation rate of hypocotyl segments increased in parallel to increasing concentrations of IAA up to 10 μM at which the elongation reached the maximum level. We observed that the elongation growth of segments treated with 10 μM IAA for 2 h was stimulated about ten fold in comparison to the water control. In addition, an indentical pattern of elongation response to IAA appeared upon 4 h incubation.

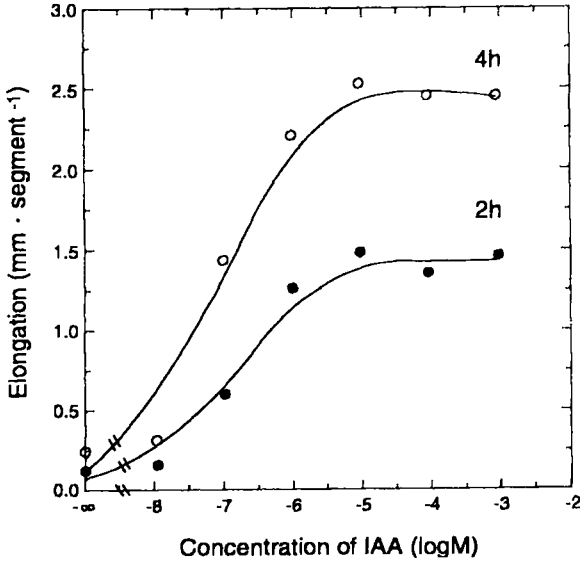

Fig. 1. Stimulation of elongation growth of soybean hypocotyl segments by IAA. Values are average from three different experiments for 1-cm hypocotyl segments (10 segments·experiment^{-1}). -∞ value on the X axis indicates the water control.

Oxygen Consumption of Plasma Membrane Vesicles

In relation to the IAA-stimulated hypocotyl elongation, we examined the activity of oxygen consumption of plasma membrane vesicles isolated from the same hypocotyl segments. Due to the impermeability of NADH to cell membranes, it has been described that the oxidation of external NADH at plasma membrane vesicles provides evidence for existence of a redox reaction system in the plasma membrane (Crane *et al.*, 1985). The expression of oxygen consumption activity of plasma membrane vesicles can be used as a clue for confirming plasma membrane redox activities (Lin, 1982). Hence, we examined the oxygen consumption of isolated plasma membrane vesicles isolated from soybean hypocotyl segments in order to evaluate a property of their redox reactions as well

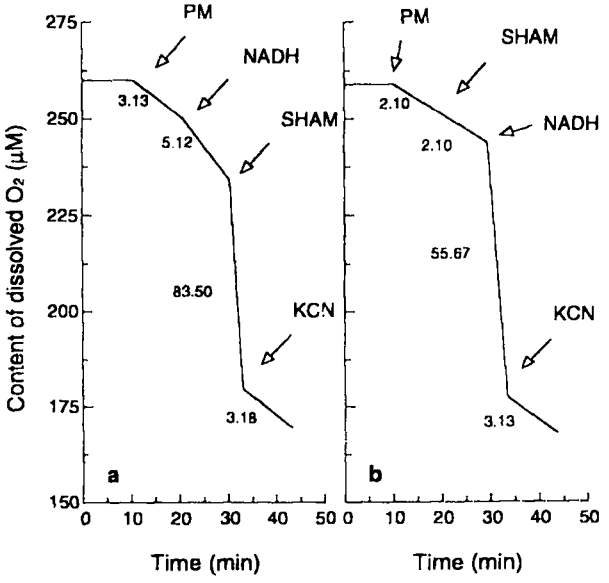

Fig. 2. Oxygen consumption by plasma membrane vesicles from soybean hypocotyls and effects of sequential addition of NADH (1 mM), SHAM (2 mM) and KCN (2 mM) on rate of O_2 consumption. NADH, SHAM and KCN were added to 0.3 mg (a) and 0.2 mg (b) membrane vesicles where indicated, respectively. The numbers indicate the slopes in nmol O_2·mg protein^{-1}·min^{-1}.

as isolation purity. It has been reported that the oxygen consumption of living organisms is largely due to mitochondrial respiration. However, a little oxygen consumption could occur upon in complete inhibition of mitochondrial respiration. This is known as residual respiration of plasma membranes (Morré, 1989). The isolated plasma membrane vesicles in this study basically consumed 3.13±0.5 nmol O_2·mg protein-1·min^{-1}. However, this oxygen consumption increased by 70% (i.e., 5.12±0.6 nmol O_2·mg protein^{-1}·min^{-1}) upon 1 mM NADH treatment (Fig. 2a).

The residual respiration and the increase of oxygen consumption can be interpreted as evidence for the existence of NADH oxidase at the plasma membrane of the soybean hypocotyl, although its increasing rate is lower than that (ca. three fold increase) of Møller and Bérezi (1985). Møller and Bérezi (1985) also reported that the NADH-stimulated oxygen consumption of plasma membrane vesicles was further increased ten to twenty fold in the presence of salicylhydroxamic acid (SHAM). In this study, the addition of 1 mM NADH followed by 2 mM SHAM into plasma membrane vesicles resulted in a twenty fold increase of oxygen consumption, which neces-

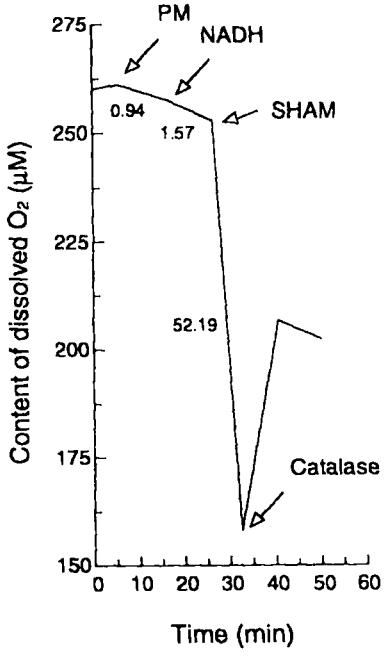

Fig. 3. Changes of dissolved O_2 level during oxidation of NADH by soybean hypocotyl plasma membrane vesicles in the presence of SHAM. NADH (1 mM), SHAM (2 mM) and catalase (16 units) were added where indicated. 0.1 mg plasma membrane vesicles were used. The numbers indicate the slopes in nmol O_2·mg protein^{-1}·min^{-1}.

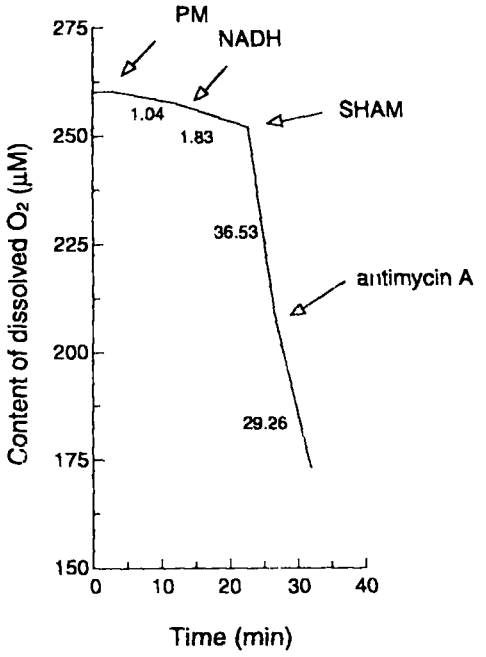

Fig. 4. Effect of antimycin A (10 μM) on the rate of NADH-dependent, SHAM-stimulated O_2 consumption by soybean hypocotyl plasmalemma vesicles. 0.1 mg membrane vesicles were used. The numbers indicate the slopes in nmol O_2·mg protein^{-1}·min^{-1}.

sarily required the presence of NADH (Figs. 2a and b). However, the rapid oxygen consumption by NADH and SHAM was reversed by a catalase treatment and H_2O_2 was produced thereafter (Fig. 3.).

Gross (1977) demonstrated that wall-localized malate dehydrogenase could produce NADH in plants, and the resultant NADH was subject to plasma membrane redox reactions leading to production of oxygen radicals and H_2O_2. He also pointed out that these oxygen radicals and H_2O_2 were linked to cell wall synthesis.

Meanwhile, the activity of oxygen consumption of soybean plasma membrane vesicles after rapid oxygen consumption by NADH and SHAM was nearly uninhibited by adding antimycin A, which is known as an NADH oxidation inhibitor in mitochondria (Fig. 4). Therefore, we were able to suggest that the isolated soybean plasma membrane vesicles were not contaminated by any mitochondrial membranes and the oxygen consumption was largely due to plasma membrane redox reactions. Furthermore, the production of H_2O_2 in plasma membranes by NADH and SHAM indicates that the plasma membrane redox activities could be involved in the processes of plant growth.

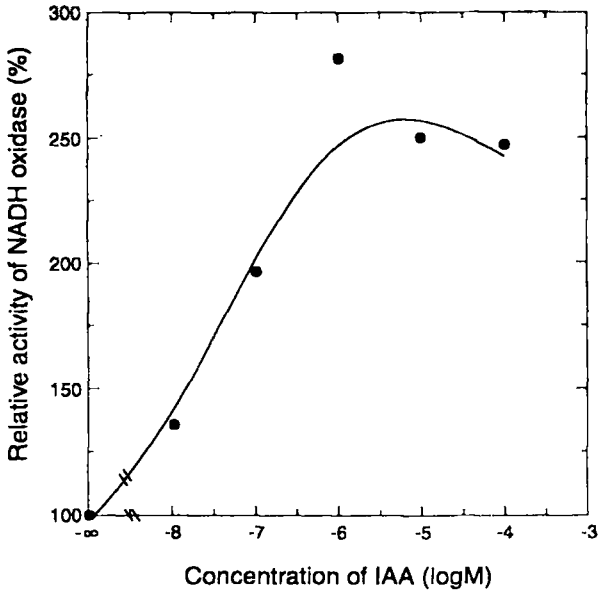

Fig. 5. Stimulation of NADH oxidase activity by IAA. The data are average values from determination with eight different plasma membrane vesicle preparations from soybean hypocotyls. Control activity (no IAA) was 12.0±2.5 nmole NADH oxidized·mg protein^{-1}·min^{-1}.

Effects of IAA on Plasma Membrane NADH Oxidase Activity

To evaluate whether plasma membrane redox reactions contribute to the IAA-induced elongation growth of soybean hypocotyl segments, the effect IAA on the NADH oxidase activity was investigated in plasma membrane vesicles isolated from the hypocotyl tissue in which the elongation was measured. The plasma membrane vesicles were preincubated with different concentrations of IAA at 25°C for 5 min before addition of NADH.

Fig. 5 illustrates that the plasma membrane NADH oxidase activity was stimulated by IAA. Furthermore, the enzyme activity increased in parallel to the IAA effect on elongation growth of hypocotyl segments. In other words, the plasma membrane NADH oxidase activity was stimulated three fold at 1 μM IAA and then reached saturation at 10 μM IAA. This result, together with a previous IAA effect on hypocotyl elongation (Fig. 1), indicates an involvement of plasma membrane redox activities in IAA-induced elongation growth.

Short-term Elongation Growth Kinetics by IAA and Plasma Membrane Redox Inhibitors

Actinomycin D and adriamycin has been known to inhibit the activity of plasma membrane redox enzymes as well as the growth of plants (Crane *et al.*, 1985). This means that the plasma membrane redox reactions may be involved in plant growth. In considering the similar patterns in the dose-response for both the elongation growth and plasma membrane NADH oxidase activities in response to IAA (Figs. 2 and 5), we could also speculate that the plasma membrane redox reactions are related to the IAA-induced elongation. Therefore, we investigated the effects of these inhibitors on short-term elongation and plasma membrane NADH oxidase activity in order to understand the regulatory mechanism of plasma membrane redox reactions by IAA.

Actually, many studies have dealt with only the plasma membrane redox activities which were involved in long-term elongation growth (i.e., over 16 h after IAA treatment) (Morré *et al.*, 1988). This long-term treatment of IAA as well as of other inhibitors

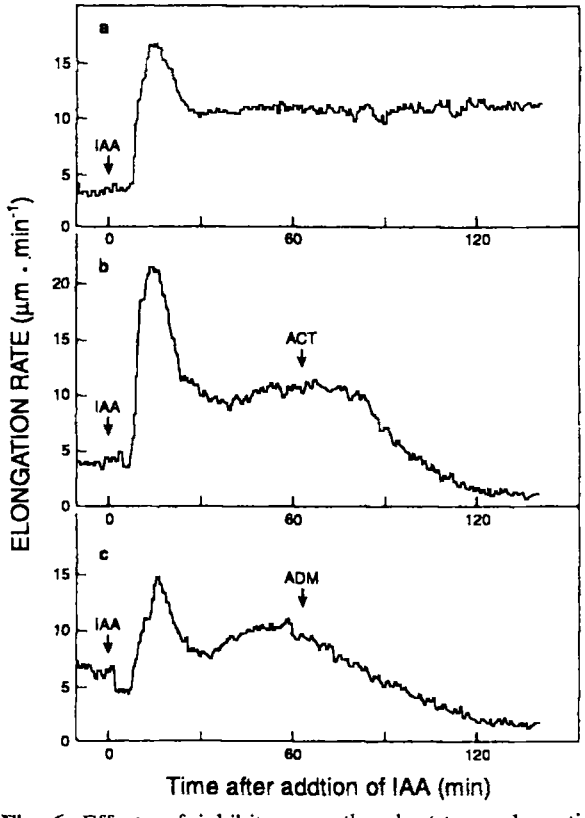

Fig. 6. Effects of inhibitors on the short-term elongation kinetics of abraded soybean hypocotyl segments. IAA (10 μM) was added 90 min after cutting the segments (=time zero); Actinimycin D (ACT) 80 μM (b) and adriamycin (ADM) 50 μM (c) were added 1 hour after IAA treatment.

may induce damage in plant tissues. In addition, recent experiments on IAA-induced elongation have focused on the short-term growth kinetics after IAA treatment (Edelmann and Schopfer, 1989). In this study, we examined the short-term elongation kinetics of hypocotyl segments by IAA or inhibitors. IAA rapidly stimulated the elongation rate of hypocotyl segments with about a 7.4+1.1 min lag time, and then the rate reached the maximum level 15 min after IAA treatment and stayed at the maxinum level for 2 hours with a small decrease (Fig. 6a). In order to see the effects of inhibitors on IAA-mediated elongation of hypocotyl segments, inhibitors (80 μM actinomycin D or 50 μM adriamycin) were added to the incubation media 60 min after IAA treatment. Actinomycin D began to decrease the elongation rate with a 20 min lag time (Fig. 6b), however adriamycin reduced the rate as soon as it was added to the incubation media (Fig. 6c). From this study, we observed that IAA and inhibitors act effectively on the elongation of segments within a short time. Such short-term effects have not been previously reported, implying that the plasma membrane could be a primary target of the actions of IAA and the inhibitors metioned above.

Activity of Plasma Membrane NADH Oxidase by Actinomycin D and Adriamycin

The effects of actinomycin D and adriamycin on the expression of NADH oxidase activity of plasma membrane vesicles were investigated to understand the regulation of plasma membrane redox systems by IAA. Plasma membrane vesicles were preincubated in the presence of 1 μM IAA with actinomycin D or adriamycin at 25°C for 5 min before adding NADH. Actinomycin D, the more potent inhibitor of plant growth, did not significantly affect NADH oxidase activities in the absence of IAA. However, it inhibited the IAA-induced activation of NADH oxidase (Fig. 7). Actually, the magnitude of inhibition of NADH oxidase activity in the presence of IAA was parallel to increasing concentrations of Actinomycin D and then the enzyme activity was inhibited by 40% at 1 mM concentration.

Hence, Actinomycin D seems to inhibit IAA actions linked to the expression of plasma membrane NADH oxidase activity. This result suggests the existence of a secondary molecule which mediates IAA signals into the plasma membrane. Meanwhile, an inhibitor of NADH-ferricyanide oxido-reductase of animal cells (Crane *et al.*, 1985), adriamycin, did not affect NADH oxidase activities of soybean plasma membranes (Fig. 8). So, the plasma membrane-associated NADH oxidase is thought to oxidize NADH regardless of the inhibition of NADH-ferricyanide oxido-reductase by

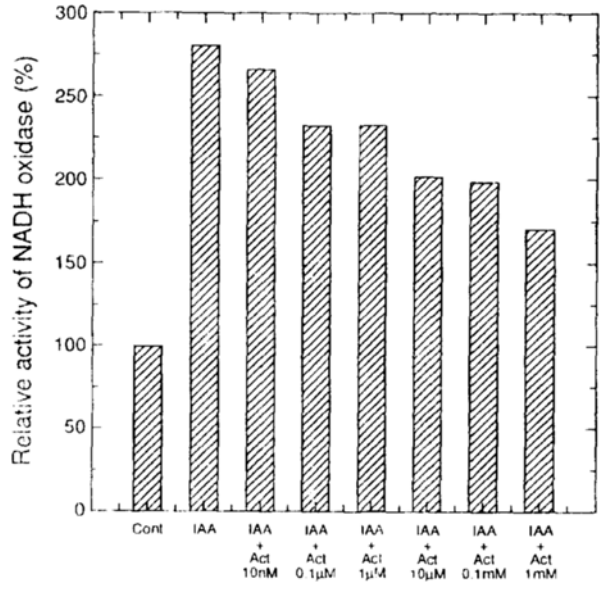

Fig. 7. Inhibition by actinomycin D on IAA-stimulated plasma membrane NADH oxidase activity from soybean hypocotyls. Actinomycin D was treated in the presence of 1 μM IAA and control activity (no IAA and no Actinomycin D) was 12.0±2.5 nmole NADH oxidized·mg protein^{-1}·min^{-1}. Cont: control, Act: actinomycin D.

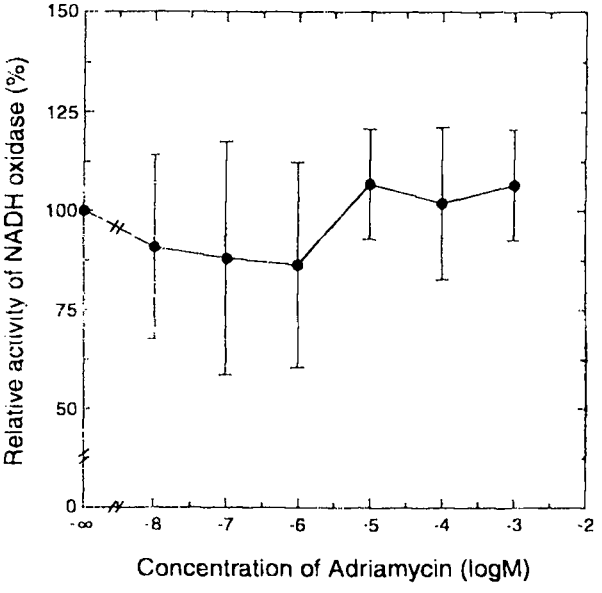

Fig. 8. Effect of adriamycin on IAA-stimulated plasma membrane NADH oxidase activity from soybean hypocotyls. Adriamycin was treated in the presence of 1 μM IAA. Control activity (no IAA and no adriamycin) was 12.0±2.5 nmole NADH oxidized·mg protein^{-1}·min^{-1}.

adriamycin. It is also believed that the IAA-regulated plasma membrane redox activity could function regardless of NADH-ferricyanide oxido-reductase expression. Moreover, the inhibition of IAA-induced elongation of soybean segments by adriamycin indicates that the IAA-regulated elongation may require unknown activity by NADH-ferricyanide oxido-reductase. In addition, the inhibition of IAA-induced elongation by adriamycin without any inhibitory effects on NADH oxidase suggests that the NADH oxidase is functionally linked to an NADH-ferricyanide oxido-reductase at the plasma membrane. In other words, the IAA-stimulated external NADH oxidase rather than the cytoplasmic NADH-ferricyanide oxido-reductase, can promote a flow of electrons from plasma membranes to the cell wall and then the oxidation of cytoplasmic NADH seems to increase by an NADH-ferricyanide oxido-reductase (Crane, 1989). The resultant protons by NADH oxidation may serve as an energy source for IAA-induced H^+ excretion and elongation.

In conclusion, the redox reaction system of isolated plasma membrane vesicles from soybean hypocotyl segments can be involved in IAA-induced hypocotyl elongation and NADH oxidase could be the key element to IAA-regulated plasma membrane redox activities.

ACKNOWLEDGEMENT

This work was supported by Seoul National University Daewoo Research Fund (94-06-2059).

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Received November 7, 1997
Accepted December 3, 1997