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Activation of Plasma Membrane NADH Oxidase Activity by Products of Phospholipase A

Andrew O. Brightman, Xiao Zhong Zhu, and D. James Morré*

Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

An auxin-stimulated NADH oxidase activity (NADH oxidase I) of plasma membrane vesicles, highly purified by aqueous two-phase partition from soybean (*Glycine max* Merr.) hypocotyls was activated by lysophospholipids and fatty acids, both products of phospholipase A action. The activation of NADH oxidase activity occurred slowly, suggesting a mechanism whereby the lipids acted to stabilize the enzyme in a more active configuration. In contrast to activation by lipids, the activation by auxin was rapid. The average K_m of the NADH oxidase after activation by lipids was four- to fivefold less than the K_m before activation. The V_{max} was unchanged by activation. The increases occurred in the presence of detergent and thus were not a result of exposure of latent active sites. Also, the activation did not result from activation of a peroxidase or lipoxygenase. Fatty acid esters, where growth promoting effects have been reported, also activated the auxin-stimulated oxidase. However, the auxin stimulation of NADH oxidase I did not appear to be obligatorily mediated by phospholipase A, nor did inhibitors of phospholipase A_2 block the stimulation of the oxidase by auxins.

NADH oxidase is one of several redox activities found in the plasma membrane of plant cells (3). Two types of NADH oxidase activity have been identified and designated NADH oxidase I and NADH oxidase II (1). The activity of NADH oxidase I, both in isolated plasma membranes and in the purified enzyme, is stimulated by auxin (2, 12). Also, NADH oxidase I shows a strong correlation with elongation growth. Elongation and NADH oxidase activity are affected similarly by inhibitors, activators, and osmotic stress (1, 13).

More than 30 years ago, the growth promoting effect of fatty acid esters on pea sections was documented (25). The conclusion was that the fatty acids were increasing the sensitivity of the pea sections to auxin (IAA) because the fatty acid esters were only effective in the presence of auxins. Methyl and ethyl esters of fatty acids (C_{14} or greater) increased elongation at concentrations ranging from 10 to 100 μM (27). The explanation put forth was that fatty acid esters and a number of related compounds were activating an electron transfer system somehow involved in auxin-stimulated growth. This idea was supported by the stimulation of O_2 uptake of pea sections by 40 μM methyl myristate (26). However, respiration (O_2 uptake and phosphorylation) by mitochondria was shown not to be affected by these lipids (16).

The present work was to determine if the growth promoting action of fatty acids and lysolipids, both products of phospholipase A activity, could be due to an activation of the auxin-regulated NADH oxidase I. The results provide evidence that growth stimulatory lipids activate NADH oxidase I, but in a manner different from that by which the auxins stimulate the activity.

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max* Merr.) seedlings were grown 4 to 5 d in darkness. Hypocotyl segments were excised under diminished light 5 mm below the cotyledon and used for growth experiments (1 cm) or for isolation of plasma membrane (2 cm).

Plasma Membrane Isolation

Plasma membranes were isolated from an 8,000 to 50,000*g* pellet (microsomal membrane fraction) using aqueous polymer phase partition (17).

Growth Assay

Segments were incubated (10/dish) in 2 mL solutions containing water (fatty acids, lysoPC[1]) or a basal medium of 1% sucrose and 50 μM $CoCl_2$ (fatty acid methyl esters) in the presence or absence of 10 μM 2,4-D and additives. After a 16 h incubation in the dark, segments were measured to the nearest 0.1 mm. Values are averages of three experiments ± SD among experiments. For growth, measurements were of 10 1-cm sections/treatment in each experiment.

NADH Oxidase Assay

NADH oxidase activity was determined using a DW2000 spectrophotometer (SLM-Aminco) in the dual wavelength mode as the decrease in absorbance at 340 nm with 430 nm as reference. Rates were recorded with an attached HP plotter/printer. Reactions containing 20 mM Tris-Mes, pH 7, 300 μM NADH, and 1 mM KCN were at 25°C with constant stirring. Plasma membrane (50–100 μg) was added to start the reactions and lysolipids and fatty acids were added after a basal rate was established.

[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; F68, Pluronic F68.

Fatty Acid Emulsion

Stock emulsions were prepared by injecting 250 μL of fatty acid ester dissolved in acetonitrile containing 4 mg of F68 (Fluka) through a 23 G × 1 inch needle from a 1 mL syringe into 10 mL of 0.04% Pluronic F68 dissolved in water. Fatty acid esters were at a final stock concentration of 2 mM with F68 at 0.08% as described (27).

RESULTS

When incubated in the presence of lysolipids or free fatty acids, NADH oxidase activity of isolated plasma membrane vesicles of soybean was activated in a time-dependent fashion over 30 min (Fig. 1). The different lysophospholipids, lyso PC, lyso PE, and lyso PI, were of nearly equal efficacy (Table I). Linoleic acid, however, was more effective than either palmitic or stearic acid in stimulating the NADH oxidase, in both the methyl ester and the fatty acid form (Table I). Equivalent stimulations of NADH oxidase were obtained with the addition of phospholipase A_2 to membranes (Table I).

The plasma membrane fractions isolated by aqueous two-phase partition from soybean hypocotyls have been extensively characterized. Two marker enzymes, a K^+-stimulated, vanadate-inhibited increment in Mg^{2+}-ATPase activity and a glucan synthetase exhibiting a high K_m for glucose, glucan synthetase II, were enriched in the fractions. The fractions were monitored by EM and, routinely, more than 90% of the vesicles were stained with phosphotungstic acid at low pH, as

Table I. *Stimulation of NADH Oxidase Activity of Plasma Membrane by Exogenous Phospholipase A, Lysolipids, and Fatty Acids*

After a basal rate of NADH oxidase activity was determined, activators were added to the reaction and the maximal stimulated rate was determined. Phospholipase A_2 (*Naja mocambique*/Sigma) was added with 100 μM $CaCl_2$ required for lipase activity. Lysolipids were added directly at the concentrations indicated. Fatty acids (methyl ester and free acids) were added from a water emulsion made 2 mM in 0.08% Pluronic F68. The same volume of Pluronic F68 alone was added for comparison. Control activity was 1.50 ± 0.07 nmol/min·mg protein.

Additive	Concentration	NADH Oxidase Activity
		% of control rate
Phospholipase A_2	4 μg/mL	
− $CaCl_2$		121
+ $CaCl_2$		270 ± 56
LysoPC	10 μM	285 ± 3
LysoPE	10 μM	270 ± 110
LysoPI	10 μM	265 ± 55
Linoleic acid		
Free acid	40 μM	225
Methyl ester	40 μM	235
Palmitic acid		
Free acid	40 μM	160
Methyl ester	40 μM	205
Steric acid		
Free acid	40 μM	135
Methyl ester	40 μM	185
Pluronic F68		140 ± 10

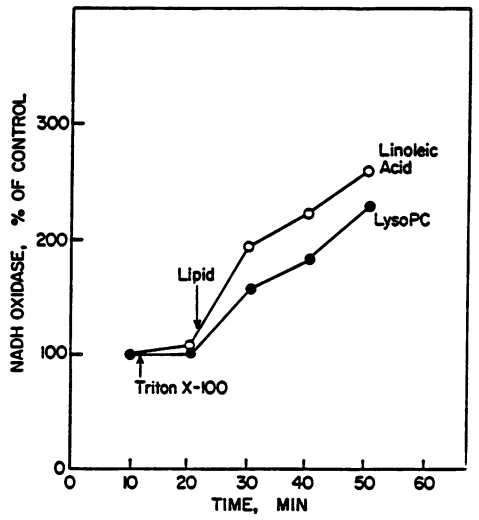

Figure 1. Stimulation of NADH oxidase of soybean plasma membranes by lysophosphatidylcholine (LysoPC) and by free fatty acid (Linoleic Acid). LysoPC (40 μM) or linoleic acid (40 μM) were added to the reaction 10 min after 0.1% Triton X-100 was added to permeabilize the plasma membranes. The hypoosmotic reaction medium contained 20 mM Tris-Mes, pH 7, 1 mM KCN, 300 μM NADH, and approximately 100 μg of plasma membranes in the absence of sucrose or salts. Oxidation of NADH was monitored continuously and the increases in activity over the control rates were reported at 10 min intervals. The time-dependent activation of NADH oxidase activity by both lysophosphatidylcholine and linoleic acid was linear for over 30 min.

is characteristic of plant plasma membranes. Markers for tonoplast (nitrate-inhibited ATPase), endoplasmic reticulum (NADH-Cyt *c* reductase), Golgi apparatus (IDPase), and mitochondria (Cyt *c* oxidase and succinate-INT-reductase) were not enriched in the fractions compared to the starting homogenate. Whereas markers for plasma membrane activities were enriched three- to fivefold compared with the postmitochondrial homogenate, the markers for mitochondria were depleted by a factor of 20. Morphologically identifiable mitochondria or mitochondrial membrane fragments (either from thin sections of fixed and embedded fractions or from preparations with 1% sodium phosphotungstate, pH 7.2) were absent.

NADH oxidase activity was increased two- to threefold by membrane permeabilizing concentrations of the nonionic detergent Triton X-100 under isoosmotic (0.25 M sucrose) conditions (Fig. 2A). However, with plasma membrane vesicles made leaky by hypoosmotic conditions (no sucrose), Triton X-100 increased NADH oxidase activity only 25%. Auxin stimulated the NADH oxidase activity both in the presence and absence of 0.1% Triton X-100 (Fig. 2B). Both lysophospholipids and free fatty acids as well as phospholipase A_2 stimulated the oxidase to the same extent either in the presence or in the absence of the detergent Triton X-100 (Table II).

Lysophosphatidylcholine added to the incubation medium of elongating sections of soybean hypocotyls resulted in small and insignificant growth stimulations at concentrations above 100 μM both in the presence or absence of 2,4-D (Fig. 3A).

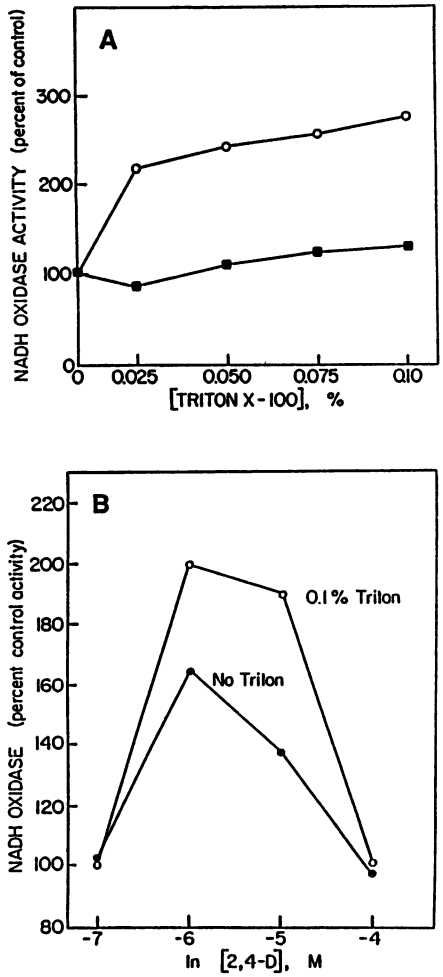

Figure 2. NADH oxidase activity in the presence of detergent. A, NADH oxidase activity was increased two- to threefold by membrane permeabilizing concentrations of the nonionic detergent Triton X-100 under isoosmotic conditions (0.25 M sucrose) (upper curve, ○). However, Triton X-100 increased NADH oxidase activity only approximately 25% in plasma membrane vesicles made "leaky" under hypoosmotic conditions (no sucrose) (lower curve, ■). Thus, the detergent was not directly activating the enzyme, but rather allowing access of the impermeant substrate, NADH. Control activities were 0.35 and 0.8 nmol/min·mg protein in the presence and absence of sucrose, respectively. B, Auxin (2,4-D)-stimulated NADH oxidase activity over a range of concentrations in the absence and presence of 0.1% Triton X-100.

Lysophosphatidylcholine stimulated the NADH oxidase activity of isolated plasma membranes either in the presence or absence of auxin with maximum stimulation at 10 μM (Fig. 3B). Free fatty acids were active in stimulating the oxidase (Table I) but were only marginally effective in stimulating growth (Fig. 4A, B).

In contrast to the free fatty acids (Fig. 4), the methyl esters of the fatty acids stimulated both the NADH oxidase and growth (Fig. 5). The optimum concentration of the fatty acid methyl esters for stimulation of growth (Fig. 5A) was higher than that for stimulation of the NADH oxidase (Fig. 5B).

When a series of fatty acid methyl esters was examined, growth was greatest with methyl linoleate (Fig. 6A). Similarly, NADH oxidase showed the greatest stimulation by methyl linoleate compared with the other fatty acid methyl esters (Fig. 6B). Comparisons included methyl palmitate, methyl stearate, and the synthetic ester F-68.

The NADH oxidase activity stimulated by lipids appeared to be neither a peroxidase nor a lipoxygenase. Incubation with superoxide dismutase or catalase did not reduce the stimulatory effects of lipids (Table III).

Compared to the lysophospholipids, the auxin response was rapid (Fig. 7). Maximum stimulation by auxin was attained in less than 5 min, whereas 10 to 30 min or longer was required for the response by lysophospholipids. Also, the stimulations by lipids and auxins were additive and could be provided in either order to achieve stimulation (Fig. 8).

Kinetic parameters measured in the presence or absence of 20 M lysophosphatidylcholine revealed a four- to fivefold decrease in the K_m of the activated form compared with the form of the activity prior to lysolipid addition (Table IV). The average V_{max} was only slightly increased by lysophospholipid addition.

Inhibitors of phospholipase A_2 did not reduce the effect of the auxin on the NADH oxidase activity of isolated plasma membrane vesicles (Table V). The substances either were without effect or were stimulatory.

DISCUSSION

Recently, an NADH oxidase activity of the plasma membrane of soybean has been shown to be stimulated by auxin and active auxin analogs in a manner that parallels their effect on elongation growth (2, 12, 13). This auxin-stimulated NADH oxidase was cyanide resistant and has been designated NADH oxidase I to differentiate the activity from NADH oxidase II, which was not hormone sensitive and was inhibited by cyanide (1). The stimulation of NADH oxidase I by products of phospholipase A_2 now has been characterized and compared to the auxin stimulation of this enzyme. Although both lysolipids and fatty acids have been reported to modulate the activity of several enzyme activities of the higher plant

Table II. *Stimulation of NADH Oxidase by Phospholipase A_2 and Lipase Products Was Not Due to a "Detergent" Effect*

Activation of NADH oxidase activity was determined in the absence and presence of a membrane permeabilizing concentration of Triton X-100 (0.1%). Linoleic acid was not added from a stock emulsion in this experiment. Control rate of NADH oxidase was 1.83 ± 0.04 nmol/min·mg protein.

Additive	Concentration	NADH Oxidase Activity
		% of control rate
Phospholipase A_2	4 μg/mL	
− Triton X-100		263
+ Triton X-100		215
LysoPC	8 μM	
− Triton X-100		220
+ Triton X-100		230
Linoleic acid	270 μM	
− Triton X-100		180
+ Triton X-100		265

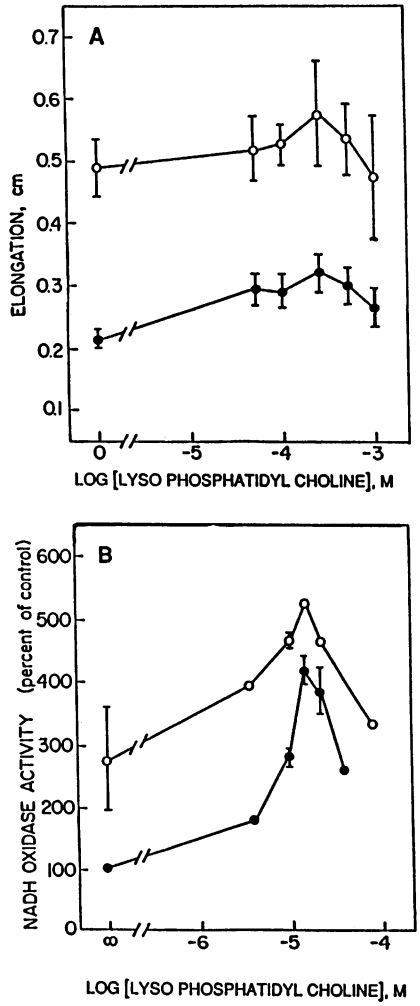

Figure 3. Elongation growth and NADH oxidase activity stimulated by lysoPC. A, Elongation of 1 cm segments was stimulated 40 to 60% by lysoPC in the absence (●) and presence (○) of 10 μM auxin (2,4-D) with maxima at 250 μM. B, LysoPC stimulated NADH oxidase activity of isolated plasma membrane over a range of concentrations with maximum stimulations at 15 μM in the absence (●) and presence (○) of 10 μM auxin. Control activity was 0.43 nmol/min·mg protein.

plasma membrane, including ATPase and proton transport activities (14, 15), neither the mechanism nor the physiological role of these activations are known.

Both lysolipids and fatty acids induced nearly linear increases in activity of NADH oxidase I over a period of 30 min. Activations of two- to threefold were common after 30 min and were occasionally as high as five- to sixfold. Similar activations were induced by incubating plasma membranes in the presence of phospholipase A_2 + calcium, indicating that indigenous lipids were equally effective as activators of NADH oxidase I activity once they were cleaved by phospholipase. The nearly equivalent capacity for activation of NADH oxidase I by the different lysophospholipids, lysoPC, lysoPE, and lysoPI, suggested that the head group was not a critical factor. In contrast, the consistently higher levels of activation by methylinoleate compared to palmitate and stearate suggested that chain length may play an important role in enzyme modulation.

The two- to threefold activation of the plasma membrane ATPase by lysophospholipids reported by Palmgren *et al.* (15) appeared to depend on chain length, degree of saturation, and head group of the lysophospholipid. Maximum stimulation was given by palmitoyl 16:0 lysophosphatidylcholine and oleoyl 18:1 lysophosphatidylcholine (15).

The possibility that the increase in NADH oxidation was due to turning on other enzymes in the plasma membrane such as peroxidase or lipoxygenase was ruled out by the lack of effect of either catalase or superoxide dismutase on the stimulated NADH oxidation. That the enzyme activity was assayed in the presence of 1 mM cyanide was further evidence against a peroxidase or lipoxygenase involvement.

Lysophospholipids have detergent-like properties when incubated with biological membranes (15). Because NADH cannot cross intact membranes, an increase in enzyme activity could be produced by increasing substrate availability by permeabilizing the membrane. Incubation of plasma mem-

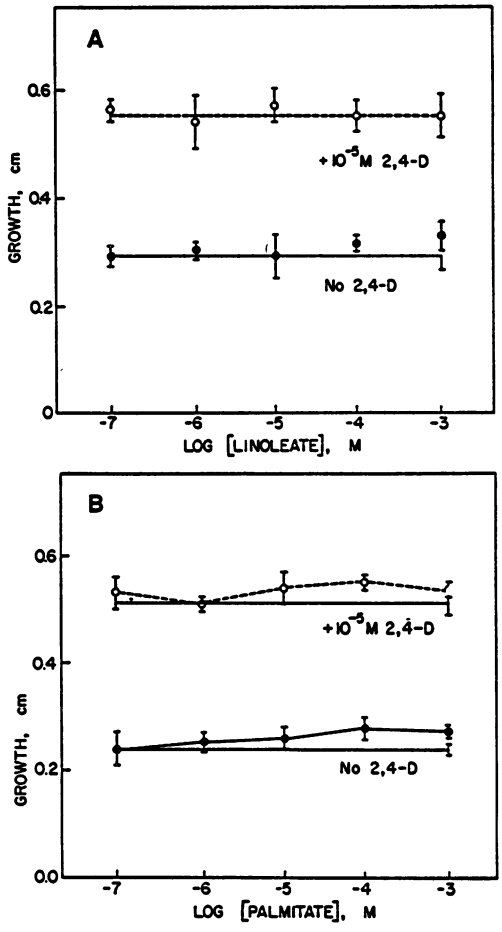

Figure 4. Growth responses to increasing concentrations of free fatty acids. A, Linoleic acid. B, Palmitic acid. Stock solutions were prepared as aqueous emulsions or dissolved by heating to 80°C and diluted with water just prior to adding the stem segments. Growth was measured in the absence (●) or presence (○) of 10 μM 2,4-D. The reference lines are growth in the absence of fatty acids.

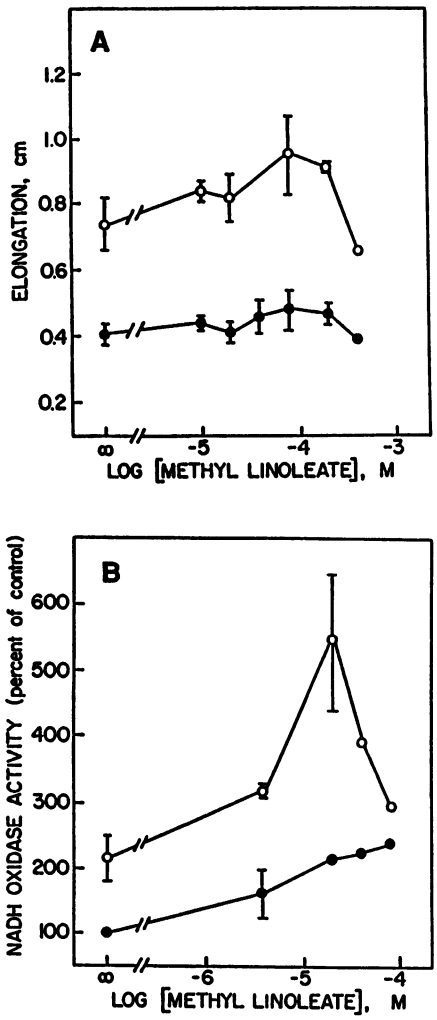

Figure 5. Elongation growth and NADH oxidase activity stimulated by methyl linoleic acid. A, In the presence of (○) of 10 μM auxin (2,4-D), elongation of 1 cm hypocotyl segments was increased up to 50% by addition of fatty acid (methyl esters of linoleic acid in a stable emulsion). A small increase was observed in the absence (●) of auxin. Optimal concentrations for both effects was 80 μM. B, NADH oxidase also was stimulated by the fatty acid. The increase, more than fivefold in the presence of auxin (○), was maximal at 20 μM. In the absence of auxin (●), the activity (1.36 ± 0.74 nmol/min·mg protein) was increased up to 2.4-fold in the presence of fatty acid.

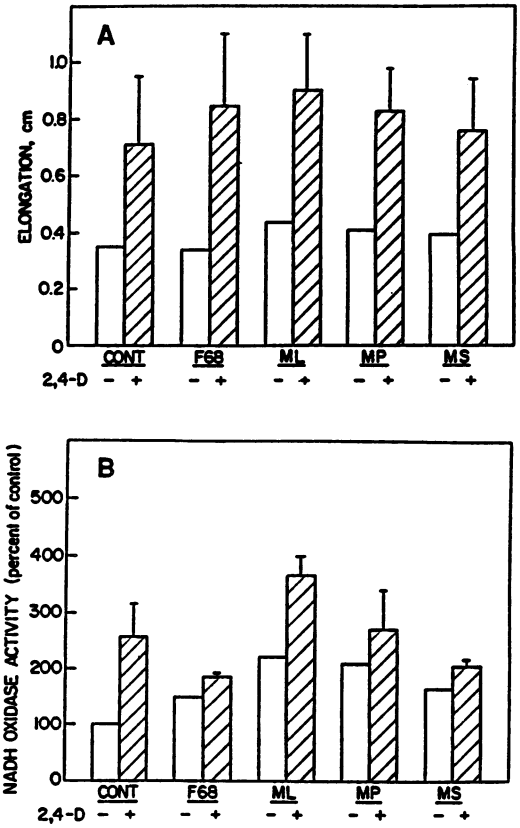

Figure 6. Elongation growth and NADH oxidase activity stimulated by several fatty acid methyl esters in the presence or absence of auxin (10 μM 2,4-D). Both elongation of hypocotyl segments (A) and NADH oxidase activity of isolated plasma membrane (B) were stimulated by 20 μM fatty acids over controls. The methyl esters of the fatty acids showed the same pattern of effectiveness on both elongation and NADH oxidase with methyl linoleate > methyl palmitate > methyl stearate. Control activity of NADH oxidase was 0.91 ± 0.14 nmol/min·mg protein.

brane in isoosmotic medium with 0.1% Triton X-100 did increase the activity of NADH oxidase two- to threefold. A similar increase was seen when the plasma membranes were shifted to hypoosmotic medium. However, in the hypoosmotic medium, only a 25% increase in NADH oxidase activity was observed with 0.1% Triton X-100. In contrast, lysolipids and fatty acids increased enzyme activity two- to fourfold even in the presence of 0.1% Triton X-100, ruling out a detergent-like effect as a major mechanism contributing to activation by lipids.

The increase in affinity of NADH oxidase I for substrate (NADH) as evidenced by the four- to fivefold decrease in K_m in the presence of lysoPC (20 μM) would imply that the

Table III. *Stimulation of NADH Oxidase Activity by Lysolipids Does Not Involve Activation of Peroxidase Activity*

NADH oxidase activity of isolated plasma membrane was determined before and after (10 min) addition of peroxidase inhibitors. Control rates (0.77 ± 0.04 nmol/min·mg protein) were in the absence of lysoPC.

Additive	Concentration	NADH Oxidase Activity
		% of control rate
LysoPC	15 μM	592
+ Superoxide dismutase	0.1 mg/mL	677
LysoPC	15 μM	460
+ Catalase	0.1 mg/mL	540

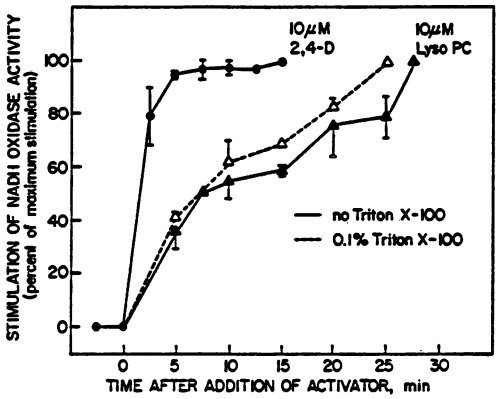

Figure 7. Time course of stimulation of NADH oxidase by auxin and lysolipid. Maximum stimulation of NADH oxidase by auxin was reached within 5 min of addition of the growth regulator to the enzyme reaction. In contrast, lysophosphatidylcholine activated the enzyme more slowly than auxin and reached a maximum in approximately 30 min of incubation (Fig. 8). Incubation in the presence of 0.1% Triton X-100 to permeabilize the plasma membrane did not increase significantly the activation rate by lysoPC. Control activity was 0.58 ± 0.06 nmol/min·mg protein.

activation was due to an alteration in enzyme conformation. Although speculative, this idea has been proposed also for enzymes of the mammalian plasma membrane. The modulations of ATPases of animal membrane systems by lysoPC, free fatty acids, or phospholipase A_2 have been well established (5, 7, 18, 23, 28–30). The H^+/K^+-ATPase of gastric mucosa is stimulated by lysoPC (5) as are the rates of Ca^{2+}-ATPases (18, 19, 30). With the Ca^{2+}-ATPase, the products of phospholipase A_2 are thought to stabilize the enzyme complex in a more reactive conformation (19).

Scherer and coworkers have shown that another type of lipid, platelet activating factor, an ether phospholipid (1–0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) with hormone-like properties in animals (4), stimulated ATP-dependent proton transport activity in microsomes from zucchini and from cultured soybean cells (10, 20–22). A plant membrane lipid similar to platelet activating factor also stimulated microsomal proton transport (21, 22). A membrane-associated protein kinase from plants is activated by lysolipids as well. This activation has been suggested to be prerequisite to the activation of ATPase activity (10, 11, 22).

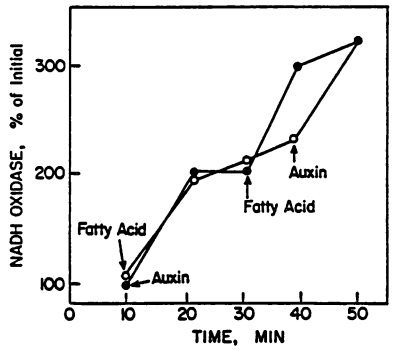

Figure 8. Stimulation of NADH oxidase by auxin and fatty acid. Plasma membrane vesicles isolated from soybean hypocotyl were assayed for NADH oxidase activity as in Figure 1. At times indicated by the arrows, auxin (10 μM 2,4-D) or fatty acids (40 μM linoleate) were added and changes in activity from control (1.97 nmol/min·mg protein) were monitored. Maximum rates over the 10 min interval are reported and show that the stimulations by auxin and fatty acid were additive and independent of the order of addition.

Table IV. *Kinetic Parameters of NADH Oxidase Activity Altered by Lysophospholipid*

NADH oxidase activity was assayed in hypoosmotic medium in the absence or presence of lysophosphatidylcholine (20 μM). Kinetic parameters were determined from measurements of NADH oxidase activity over the range of 1.5 to 450 μM NADH.

Additive	K_m	V_{max}
	μM	nmol/min·mg protein
None	70–148	1.4–2.9
LysoPC	16–32	1.5–3.3

Other membrane enzymes reported to be influenced by lysolipids include 1,3-β-D-glucan synthetase of plants (6) and sialyl- (24) and galactosyltransferase (8) activities in animals. In plants, linoleic acid has been shown to stimulate a soluble calcium-dependent protein kinase in silver beet leaves (9).

Because NADH oxidase I sensitivity to auxin parallels the sensitivity of elongating tissue (1), and because Stowe and Penny have shown that lipids can effect the rate of elongation in the presence of auxin (16, 25–27), the effects of auxin and growth active lipids on NADH oxidase were compared. The observations that sequential addition of auxin and fatty acids were additive regardless of the order of addition and, perhaps more striking, that the rate of activation of NADH oxidase was much slower for lysoPC compared with auxin, would indicate that these molecules were acting independently and at separate sites on the enzyme. Also, the lack of effect of phospholipase inhibitors on auxin activation indicates that auxin stimulation was not obligatorily mediated by lipase activity.

Table V. *Phospholipase A_2 Inhibitors Did Not Block Stimulation of NADH Oxidase by Auxin*

Plasma membrane was incubated with the inhibitors 10 to 20 min before addition of auxin. Rates in the presence of inhibitors alone were included for comparison. Control rate of NADH oxidase activity was 0.76 ± 0.24 nmol/min·mg protein.

Additive	Concentration	NADH Oxidase Activity
		% of control rate
EDTA	1 mM	164
2,4-D	10 μM	190
EDTA + 2,4-D		257
EGTA	100 μM	100
2,4-D	10 μM	155
EGTA + 2,4-D		163
$ZnCl_2$	1 mM	35
2,4-D	10 μM	190
$ZnCl_2$ + 2,4-D		190

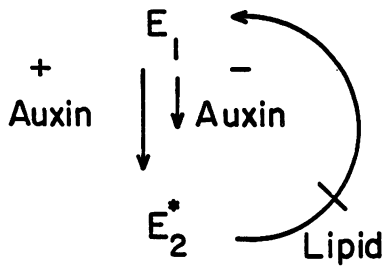

Figure 9. Model to explain the interactions of auxin and lipids in activation of NADH oxidase I of the plant plasma membrane. The enzyme is assumed to exist in two conformations, E_1 (not activated) and E_2 (activated). E_1 may be converted spontaneously to E_2, or the E_1 to E_2 conversion may be stimulated by auxin. The suggested role of the lipid activators is to stabilize the active E_2 form of the enzyme and delay its return to the less active, E_1, conformation.

Our working hypothesis proposes that NADH oxidase can exist in at least two forms of differing activity, as diagrammed in Figure 9. The more active forms, for example, are apparently favored with auxin treatment but might be attained spontaneously, even in the absence of auxin but with lower probability. One role of the lipid activators then would be to prevent the conversion of the activated forms of the enzyme back to the less active forms. In this manner, products of phospholipase A_2, for example, in the plasma membrane would have the potential to mimic, prolong, or amplify responses to auxin growth regulators to help explain the observed growth responses of elongating stem segments to such molecules (25–27).

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