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IN VITRO STIMULATION BY 2,4-DICHLOROPHENOXYACETIC ACID OF AN ATPase AND INHIBITION OF PHOSPHATIDATE PHOSPHATASE OF PLANT MEMBRANES

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SUMMARY

The auxin herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was shown to modulate the activities of several phosphatases with membranes isolated from soybean hypocotyls under conditions where degradative changes in the membranes were minimized. The medium for isolation of membranes consisted of 0.1 M Tris/HCl or Tris/acetate, pH 6.5, 0.5 M sucrose, 4% choline (w/w) and 4% ethanolamine (v/v) to inhibit phospholipase D, 20 mM EGTA [ethyleneglycol-bis-(β aminoethyl ether) N,N-tetracetic acid] and 1 mM nupercaine, to inhibit phospholipase A. In contrast, the inactive auxin analog 2,3-D, did not influence ATPase activity. Endogenous release of inorganic phosphate from an unidentified source was also stimulated 30% by 2,4-D. Phosphatidate phosphatase was inhibited by 2,4-D, whereas hydrolysis of glucose-6-phosphate was not influenced by 2,4-D under the same conditions. These observations may be of relevance to the proton pump hypothesis of growth regulation.

INTRODUCTION

Reports of effects or regulation of auxin hormones on plant enzymes *in vitro* have been few (1,2). Studies from many laboratories indicate that membranes are probably the primary target for the action of these hormones (3-8). Stimulation of proton extrusion by auxins (9-11) and the general requirements of ion transport for ATP suggest the possibility of a hormone-sensitive ATPase (12,13). Such an enzyme has already been described (2) although these results have been challenged (13-15).

In previous studies a marked degradation of phospholipids by phospholipase D during membrane isolation was noted. Since phospholipids are thought to be essential for auxin binding (6) a primary objective was to develop procedures to reduce phospholipid degradation during the isolation of plant membranes. In part this is reported in a separate paper (16). Here we demonstrate that

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phospholipase A can be inhibited by the local anaesthetic, nupercaine (17). Subsequently, the inhibition of phospholipid degradation enabled us to reproduce a stable in vitro auxin-sensitive membrane system from soybean hypocotyls. Effects of 2,4-D on three phosphatase activities are described.

METHODS

Isolation of membranes. Plant growth and isolation of membranes were as described (18) with suitable modifications due to the omission of coconut-milk and the use of different buffers. The hypocotyls from 4 day old etiolated beans were homogenized in 0.1 M Tris/HCl (or Tris/acetate for ATPase experiments) pH 6.5, 20 mM EGTA, [ethyleneglycol-bis-(β-aminoethyl ether) N,N-tetracetic acid], 4% choline (w/w), 4% ethanolamine (v/v), 1.0 mM nupercaine (CIBA) and 0.5M sucrose. Addition of choline and ethanolamine to all buffers prevented degradation by phospholipase D (16). The brei was filtered through cheesecloth and the suspension was centrifuged for 12 minutes at 16,000 g. The supernatant was layered on a step-gradient of layers of 4 ml 0.8 M sucrose, 4 ml 1.0 M sucrose, 4 ml 1.2 M sucrose, and 6 ml 1.4 M sucrose, in the same buffer, except that the nupercaine concentration was 0.15 mM. After centrifugation at 90,000 g for 60 minutes, the four bands at the 0.5 M/0.8 M, 0.8 M/1.0M, 1.0 M/1.2 M, and 1.2 M/1.4 M sucrose interfaces were collected with a pasteur pipette and designated I, II, III, and IV with increasing density. The fractions were diluted with buffer, making the nupercaine concentration 0.3 mM and pelleted (45 min, 90,000 g). Samples were taken for electron microscopy and the total pellets resuspended in 2-5 ml of either a buffer containing 50 mM sodium acetate, 4% choline (w/w), 4% ethanolamine (v/v) pH 5.5 with 0.15 mM nupercaine for phosphatidate phosphatase assays or in a buffer with 10 or 50 mM Tris/acetate, 4% choline (w/w) 4% ethanolamine (v/v) pH 5.5 and 0.15 mM nupercaine for ATPase assays.

Electron microscopy. Pellets were fixed in 0.1 M potassium phosphate buffer pH 7.2 containing 2.5% glutaraldehyde (18) followed by 1% osmium tetroxide in the same buffer. Plasma membrane was stained according to Roland et al. (19).

Lipids. Lipids were isolated (20) and chromatographed with two-dimensional thin layer chromatography (21). Lipid phosphorous was determined as described (22).

Phosphatidate phosphatase assay. Substrate, nupercaine, and 2,4-D were mixed in appropriate solutions in buffer so that their final concentrations were 2 mM, 135 µM, or varied as indicated in figures, respectively in a final volume of 250 µl (23). The membranes were added as a suspension in buffer with 0.15 mM nupercaine. The protein concentration in the assays was about 1.0 mg/ml protein. After mixing, the assay tubes were immediately transferred from an ice bath to a 20°C water bath for 1-2 hours. Appropriate blanks were included for each hormone concentration. The reaction was stopped by adding 1 ml of 7% trichloroacetic acid. After at least one hour at ice bath temperature, the precipitate was removed by low speed centrifugation and the total supernatant was combined with 2 ml freshly prepared phosphate reagent consisting of 6 g ascorbate, 3 ml 10% ammonium molybdate and 1.5 ml concentrated sulfuric acid in 120 ml water. After 5 min in a 37°C water bath the absorbance was read at 650 nm. Protein was determined by the method of Lowry et al. (24).

Endogenous phosphatase activity. The same assay as for phosphatidate phosphatase assays (without substrate) was used except that the protein concentration was 2.5 mg/ml.

ATPase assay. A final volume of 250 µl contained 3 mM ATP (Tris-salt), 1.5 mM $MgSO_4$, and 10 µM or 135 µM nupercaine, and 2,4-D or 2,3-D as indicated in the figures. The final protein concentration was about 0.2 mg/ml. The

Table I. Phospholipid composition of membranes isolated with or without nupercaine

Nupercaine		Total lipid phosphorous (%)			
		Frct. I	Frct. II	Frct. III	Frct. IV
Omitted in isolation	Phosphatidylcholine	48.0	44.6	34.8	30.4
	Phosphatidylethanolamine	23.2	26.8	30.2	32.6
	Phosphatidylinositol	13.7	6.2	9.4	6.8
	Phosphatidylglycerol	2.7	0.6	4.2	7.5
	Phosphatidylserine	0.6	2.2	2.9	trace
	Phosphatidic acid	3.0	3.7	4.5	4.8
	Lysophospholipids	8.8	15.9	14.0	16.3
Present during isolation	Phosphatidylcholine	56.6	51.0	40.5	35.8
	Phosphatidylethanolamine	27.1	30.7	42.8	43.5
	Phosphatidylinositol	7.5	6.6	5.3	4.4
	Phosphatidylglycerol	1.0	1.6	7.0	8.5
	Phosphatidylserine	1.3	2.1	n.d.	n.d.
	Phosphatidic acid	2.1	3.5	1.7	1.3
	Lysophospholipids	4.4	4.5	2.7	6.5

reaction was started by the addition of enzyme and immediate transfer of the assay to a 15°C water bath for 20 minutes. Stimulation by potassium was measured in the presence of 50 mM potassium chloride (25). Under these conditions endogenous phosphate release was not detected. The reaction was stopped by the addition of 1 ml of 7% trichloroacetic acid and, the precipitate was removed by centrifugation. Inorganic phosphate was determined as for phosphatidate phosphatase assays. 20 min incubation at 30°C and subsequent cooling gave best results for reproducible color development.

Acid phosphatase. Substrate concentration was 20 mM glucose-6-phosphate and protein concentrations were 50-100 mg protein/ml. Buffers, incubation conditions, and determination of phosphate were as for phosphatidate phosphatase.

RESULTS

Nupercaine greatly reduced the formation of lysophospholipids during the isolation of the membranes (Table 1). Membranes isolated with nupercaine had 3 fold lower concentrations of lysophospholipids.

In membranes isolated and incubated in the presence of nupercaine we observed a stimulation by 2,4-D of a phosphatase using ATP as a substrate (Fig. 1). Freshly isolated membranes gave best results. 2,4-D in nanomolar to micromolar concentrations stimulated Mg^{2+} ATPase activity 20-30%. Membranes from fraction II and sometimes fraction IV were most susceptible to 2,4-D. Preliminary electron microscopic data show that fraction II contained the

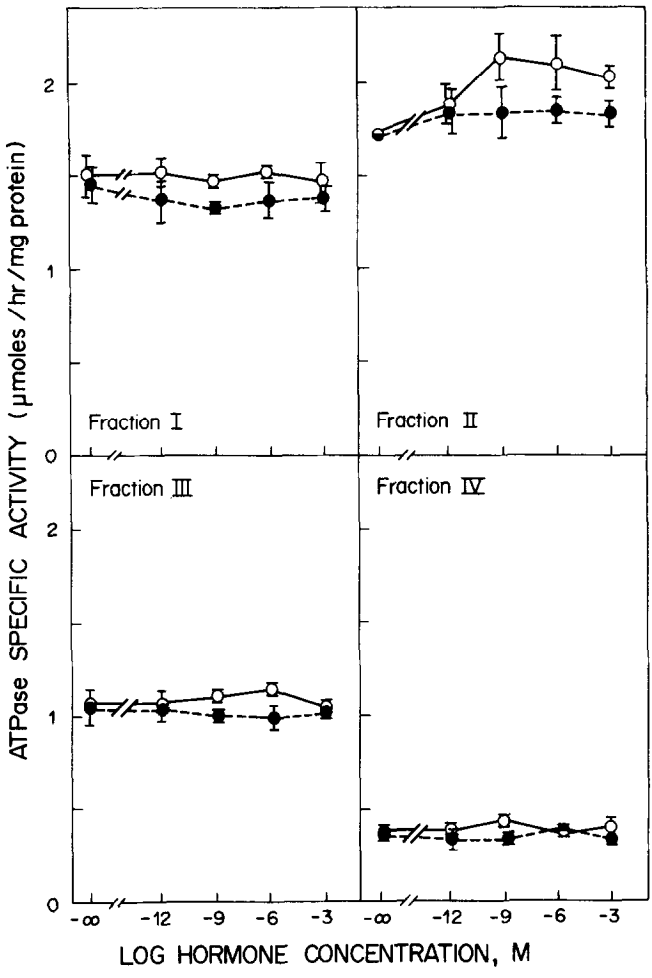

Figure 1. Modulation of ATPase activity by 2,4-D and 2,3-D at pH 5.5. Assays contained 10 mM Tris and 10 µM nupercaine. No potassium was added. Vertical bars indicate the standard deviation (n = 3). Open symbols: 2,4-D; closed symbols: 2,3-D.

highest percentage of plasma membrane. In contrast, 2,3-D stimulated ATPase activity only slightly or not at all. Under other incubation conditions (pH 6.0) similar to those employed by Hodges and Leonard (25), the stimulation was less, especially in the presence of 50 mM KCl (Fig. 2). ATPase assayed at pH 6.5 was not influenced by either 2,4-D or by 2,3-D (Fig. 3). The concentration of nupercaine (10 or 135 µM) or Tris (10 or 50 mM) seemed not to affect the stimulation whereas the variation of the pH influenced the stimulation of ATPase activity by 2,4-D rather drastically (compare Figs. 1-3).

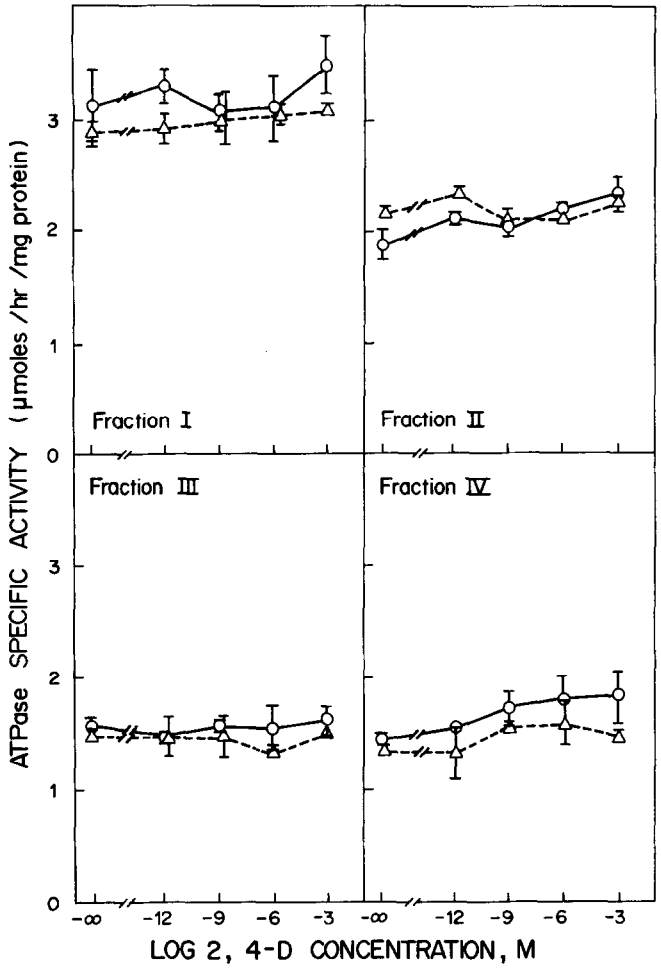

Figure 2. Modulation of ATPase activity by 2,4-D at pH 6.0. Assays contained 50 mM Tris and 135 μM nupercaine. Circles: without potassium chloride; triangles: with 50 mM potassium chloride.

Endogenous phosphate release was also observed to be stimulated up to 30% by 2,4-D over a broad range of hormone concentrations (Fig. 4). The absolute levels of the endogenous activity were variable among different experiments.

In contrast to ATPase, phosphatidate phosphatase was inhibited by 2,4-D as other phosphatase activities were stimulated (Fig. 5). The inhibition of phosphatidate phosphatase ranged from 15-25% in membranes isolated and incubated in the presence of nupercaine. Acid phosphatase activity in the same membrane preparation and under the same incubation conditions was not influ-

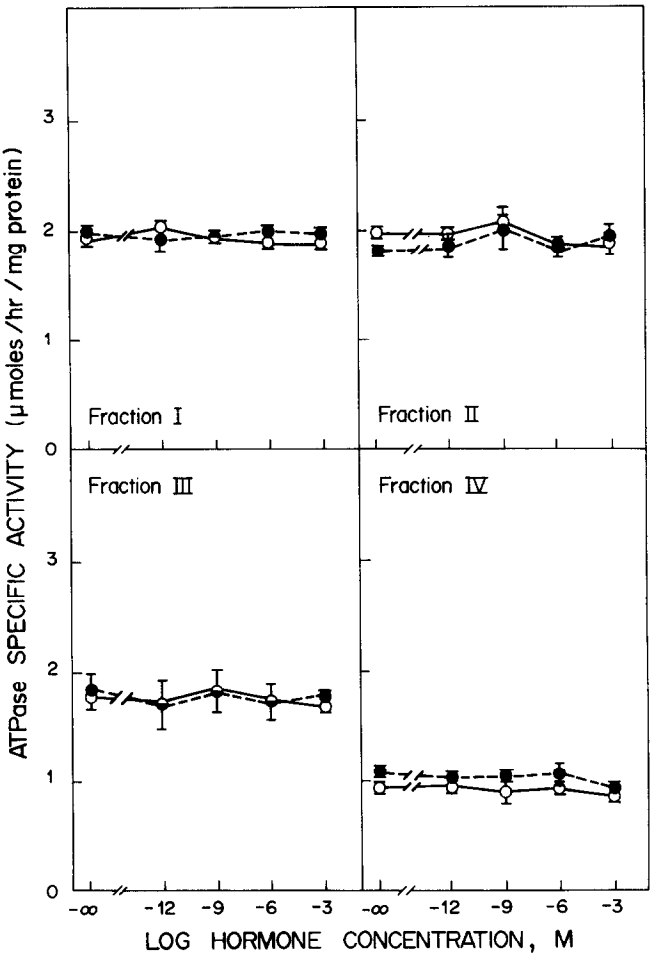

Figure 3. Modulation of ATPase activity by 2,4-D and 2,3-D at pH 6.5. Assays contained 10 mM Tris and 10 µM nupercaine. Open symbols: 2,4-D; closed symbols: 2,3-D.

enced by 2,4-D (Fig. 5b). Fraction II consistently showed the greatest response. In other experiments with a different lot of soybeans we have observed 30-70% inhibition of phosphatidate phosphatase in the absence of nupercaine. Omission of nupercaine during the isolation of the membranes resulted quite often in a failure to detect enzyme inhibition.

DISCUSSION

Our report demonstrates that the development of membrane protective procedures (Table 1 and ref. 16) allows demonstration of an auxin-sensitive in

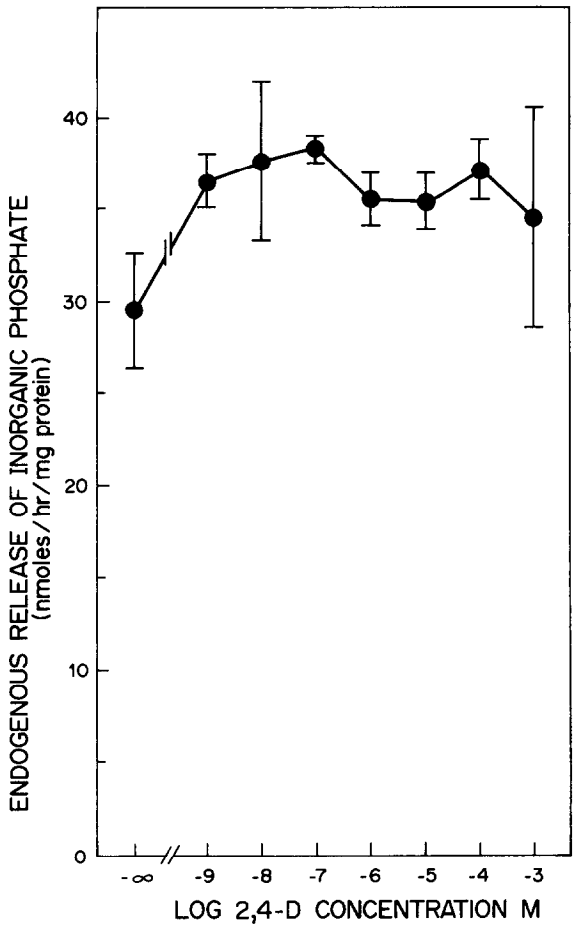

Figure 4. Stimulation of endogenous phosphate release by 2,4-D in fraction I. Vertical bars indicate the standard deviation (n = 6 for control and 10^{-6}M, 2,4-D, all others n = 3).

vitro system with soybean hypocotyl membranes. Two, or possibly three, phosphatase activities were modulated by 2,4-D *in vitro*.

ATPase activity was stimulated maximally in the presence of nanomolar and micromolar concentrations (Fig. 1). This correlated well with the dissociation constants measured for auxin *in vitro* (3,5,6,8) and the optimal growth stimulation in soybeans by 2,4-D *in vivo* (26). Since ATPase activity in the same experiment was not significantly influenced by 2,3-D it is unlikely that we observed an only unspecific "narcotic" action of 2,4-D on the membranes (27). Even though the 2,4-D effects on ATPase activity were small they were repro-

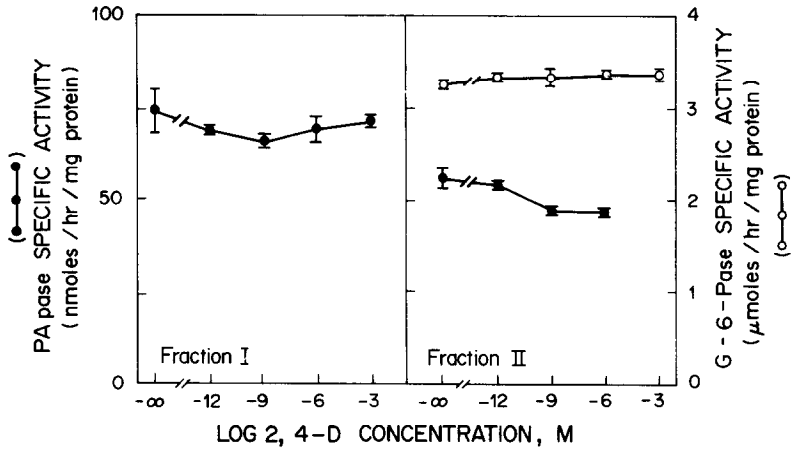

Figure 5. Inhibition of phosphatidic acid phosphatase by 2,4-D. Vertical bars indicate standard deviation (n = 3). Open symbols: acid phosphatase activity with glucose-6-phosphate; closed symbols: phosphatidate phosphatase activity.

ducible. The pH-dependence of ATPase stimulation by 2,4-D (compare Figs. 1-3) is similar as the pH-dependence of auxin-binding (5,6) with an optimum at pH 5.5. The partial separation of the auxin-binding activity from ATPase indicates that 2,4-D might exert its effect in an indirect manner (28,29). However, it is difficult to compare our data with a previous report on an auxin-stimulated and Mg^{++}-dependent ATPase (2). One important difference between our experiments and theirs was a pretreatment of the mung beans *in vivo* with 10^{-5} M IAA which resulted in a dramatic auxin-dependent increase of a Mg^{++}-stimulatable ATPase *in vitro* of over 280% (2). Without pretreatment, a 35% increase of ATPase activity due to auxin *in vitro* was observed which is comparable to our results. We did not try a pretreatment of the soybeans with 2,4-D because it would be difficult to rule out the presence of traces of 2,4-D during the isolation of the membranes and in the isolated particles themselves. Besides this, no evidence for a positive adaptation of the growth response to auxin *in vivo* has been reported. The failure of Cleland and Lomax (15) to repeat the experiments of Kasamo and Yamaki (2) is not surprising in view of our own difficulties to overcome membrane degradation (16). Their experiments employed the procedure of Hodges and Leonard (30) to isolate plasma membranes.

With this procedure, degradation by phospholipase A and phospholipase D is considerable (31).

The evidence presented here and the good agreement with other in vivo and in vitro data suggests that the stimulation of ATPase activity in vitro might be a part of the physiological response to auxin. We must emphasize, however, that a suggestion for a functional role of this ATPase activity can be only speculative at present even though a close connection between ATPase activity stimulation in vitro and auxin-stimulated ion transport in vivo is indicated (9-15).

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