AUXIN (2,4-D) STIMULATION (IN VIVO AND IN VITRO) OF POLYSACCHARIDE SYNTHESIS IN PLASMA MEMBRANE FRAGMENTS ISOLATED FROM ONION STEMS[1]

W. J. VanDerWoude, C. A. Lembi and D. James Morré

Department of Botany and Plant Pathology and Department of Biological Sciences
Purdue University, Lafayette, Indiana 47907

Received October 29, 1971

Summary

Glucan synthetase activity associated with plasma membrane of onion stem was increased by adding the auxin herbicide 2,4-D. The effect was expressed either by pre-incubating the tissue with 2,4-D (in vivo) or by adding 2,4-D to the synthetase assay (in vitro). The 2,4-D effect was most pronounced on the synthesis of polysaccharides soluble in hot water. This is the first biochemical demonstration of an in vitro response to auxin of magnitude comparable to that observed in vivo. The results show that initial responses to the hormone occur at the plasma membrane.

Lembi et al. (4) found that plasma membrane-rich fractions isolated from maize coleoptiles by low shear homogenization and differential and sucrose gradient centrifugation, specifically bound N-1-napthylphthalamic acid (NPA) a weak auxin and inhibitor of auxin transport. These results provided indirect evidence for an interaction between auxins and the membrane of the cell surface. In this paper, we report an in vitro response of a plasma membrane-associated enzyme system of plants, referred to as glucan synthetase, to the auxin herbicide 2,4-D. The response, an increased rate of polysaccharide synthesis, is direct evidence for a specific interaction between the auxin and the surface membrane of plant cells.

---

[1]This work was supported by a grant from the National Science Foundation (GB 23183), a National Science Foundation Postdoctoral Fellowship to Dr. Lembi and the Purdue University Joint Highway Research Project. Purdue University A. E. S. Journal Paper No. 4515.

Methods and Materials

Plasma membrane fractions were isolated from stems of green onions obtained commercially (Allium cepa L.) according to the procedure of Lembi et al. (4). Onion stem explants, harvested as described by Morré (5), were placed base down on circles of Whatman No. 1 filter paper moistened with 15 ml deionized water with or without 5 μM 2,4-D (Eastman) in 1 x 20 cm petri plates and pre-incubated 16-18 hr at 25°.

To isolate plasma membranes, approximately 15 g of the incubated explants were homogenized in 15 ml of a freshly prepared medium consisting of 0.1 M K_2HPO_4, 1 mM dithiothreitol and 0.02 M EDTA in coconut milk as the solvent, adjusted to pH 6.5 (= coconut milk medium) and containing 0.5 M sucrose. Nuclei and other endogenous cellular components of the coconut milk were removed by high speed centrifugation (100,000 g, 90 min) before preparing the medium. Homogenates were prepared in the cold using a mechanized razor blade chopper (6). Cell walls and unbroken cells were removed by filtration through a single layer of miracloth (Chicopee Mills, New York), and washed on the filter with an additional 20 ml of medium. The filtered homogenate (ca. 35 ml) was centrifuged in 5.4 ml tubes for 30 min at 10,000 g (Spinco L2-65B, SW 50.1 rotor) to remove nuclei, plastids, mitochondria and large membrane fragments. The supernatant containing microsomes and the majority of the cytoplasmic membrane fragments was then sedimented at 44,000 g for 30 min onto a two-layered cushion consisting of 1.5 M and 2.0 M sucrose in coconut milk medium. The supernatant was removed and replaced by a step gradient of 0.5, 0.8, 1.0, 1.2 and 1.3 M sucrose in coconut milk medium. The gradient was then centrifuged for 90 min at 100,000 g. Plasma membranes were recovered from the 1.0/1.2 M sucrose interface of the gradient, resuspended in homogenization medium and pelleted for 30 min at 100,000 g. The plasma membrane fragments were identified by electron microscopy after differential staining with a mixture of phosphotungstic acid and chromic acid which specifically and characteristically stains the plant plasma membrane (4, 9, Lembi et al., in preparation).

The glucan synthetase assay is adapted from that of Ordin and Hall (10) and is based on the incorporation of isotopically labeled sugars from appropriate sugar nucleotides into polysaccharides of varying solubility. The substrate concentration was increased to saturate the substrate requirements for the synthetases involved with glucose incorporation into lipid soluble compounds and intermediates (12). The lipid-soluble activity of onion stem is characterized by a high affinity for UDP-glucose (K_m = 2.5 X 10^{-8}M) and competes with the synthesis of lipid-insoluble glucans at low substrate concentrations (Lembi et al., in preparation). As shown previously by Ordin and Hall (11) and Feingold et al. (3) predominantly β-1,3-linkages are produced from UDP-glucose at these high substrate concentrations.

Fractions for assay were resuspended in 0.1 M tris-HCl containing 4 mM disodium EDTA and 1 mM dithiothreitol, final pH 8.0, at a concentration of 1-4 mg protein/ml. The assay medium contained 0.3 mμmoles UDP-glucose-C^{14} (227 μC/μmole), 200 mμmoles unlabeled UDP-glucose, 10 μmoles Tris-HCl (pH 8.0), 0.4 μmoles disodium EDTA, 0.1 μmole dithiothreitol, 4 μmoles $MgCl_2$, 2 μmoles cellobiose as the acceptor and an aliquot of the fraction to provide 50-200 μg protein in a final volume of 200 μl at a final pH of 8.0. Assays were incubated 15 min at 25° on a reciprocating water bath-shaker and the reaction was stopped by heating in a boiling water bath for 5 min.

Prior to fractionation of the synthesized polysaccharide into hot water soluble-, lipid soluble-, sodium hydroxide soluble- and sodium hydroxide insoluble-constituents, cellulose powder (Sigmacell, Sigma Chemical Company, St. Louis, Missouri), 1-2 mg, was added to each assay tube. The mixture was

then extracted 3 times, 5 min each, with 1 ml aliquots of water at 95-100°. Insoluble polysaccharides were recovered after each extraction by centrifugation at 25,000 g for 12 min. Hot water soluble polysaccharides were recovered from the hot water extracts by addition of 10 mg Ficol (mol. wt. 400,000, Pharmacia, Uppsala) followed by precipitation from cold 70% ethanol and centrifugation (25,000 g for 30 min). The resulting pellet was washed once with cold 70% ethanol, solubilized in hot water and evaporated to dryness on planchets for direct determination of radioactivity.

The hot water insoluble residue remaining after hot water extraction was fractionated further in some experiments (Table III). To remove lipid-soluble materials, the residue was extracted once with 1 ml of methanol-chloroform (2:1, v/v) and once with 1 ml of absolute methanol. The methanol-chloroform and methanol extracts were combined and evaporated on planchets to provide a measure of the synthesis of lipid soluble sugar derivatives and intermediates (12). After removal of lipids, the residue was then extracted twice with 0.25 ml aliquots of hot 1 N sodium hydroxide for 5 min followed by 0.75 ml of cold water. The combined alkali extracts (including the water wash) were dried on stainless steel planchets. The alkali insoluble residue corresponds to the classical "cellulose" fraction but in these studies consists primarily of β-1,3-linked glucans. Self-absorption corrections were determined and applied to each of the fractions.

Results

The specific activity of a marker enzyme system UDP-glucose: glucose glucosyltransferase (glucan synthetase), concentrated in plasma membrane fractions isolated from onion stem explants (Lembi *et al.*, in preparation), is markedly increased by incubating the explants in the presence of the

TABLE I

IN VIVO STIMULATION OF PLASMA MEMBRANE-ASSOCIATED GLUCAN SYNTHETASE BY 2,4-D PRETREATMENT OF ONION STEM TISSUE

Onion stem explants (5) were incubated for 16-18 hours at 25° ± 5 μM 2,4-D. Plasma membrane fractions were isolated by the method of Lembi *et al.* (4). Units of specific activity of the isolated fractions are mμmoles glucose incorporated into total hot water insoluble polysaccharide/hr/mg protein

Tissue preincubation	Specific activity Expt. I	Expt. II	Expt. III	Expt. IV
None	319	321	384	324
5 μM 2,4-D	410	391	458	410
Δ 2,4-D %	29	22	19	26

synthetic plant growth regulator 2,4-D (Table I). Tissue was incubated for 16-18 hours in the presence or absence of 5 μM 2,4-D and the cell-free synthesis of hot water insoluble glucans by isolated plasma membrane fractions was determined. The specific activity of the glucan synthetase of plasma membranes isolated from the 2,4-D-incubated tissues was increased approximately 25% over that of plasma membranes from control tissues incubated in the absence of 2,4-D.

A similar response results from the *in vitro* addition of the auxin. Plasma membranes were isolated from tissues incubated in the presence or absence of 5 μM 2,4-D and each of the preparations was then assayed in the presence or absence of 5 μM 2,4-D added to the enzyme assay medium (Table II).

TABLE II

IN VITRO 2,4-D STIMULATION OF PLASMA MEMBRANE-ASSOCIATED GLUCAN SYNTHETASE COMPARING ONION STEM TISSUE PRE-INCUBATED ± 2,4-D

Plasma membrane fractions were isolated from stem explants and units of specific activity are as described in Table I. 2,4-D (5 μM) was then added to the enzyme assay to test for *in vitro* stimulation of the membrane-associated synthetase.

Auxin added to Tissue incubation	Synthetase assay	Expt. II	Expt. III	Expt. IV	Expt. V	Expt. VI	Ave.
None	None	321	384	324	342	377	350
	5 μM 2,4-D (A)	344	443	373	366	414	388
	Δ 2,4-D, %	+7	+15	+15	+7	+10	+11
5 μM 2,4-D	None	391	458	410	---	---	420
	5 μM 2,4-D (B)	401	483	393	---	---	426
	Δ 2,4-D, %	+3	+5	-4	--	--	+1
	B/A	1.16	1.09	1.05	--	--	1.10

A consistent stimulation of 5-15% was observed in the synthesis of hot water insoluble glucans when 2,4-D was added to plasma membranes isolated from

control tissues. With plasma membranes isolated from 2,4-D-incubated tissues, incubation with 2,4-D had little or no additional effect on the glucan synthetase activity (Table II).

The products of the synthetase assay are heterogeneous and not all fractions are affected similarly by the presence of 2,4-D in the enzyme assay. After fractionation, the largest *in vitro* 2,4-D response of the hot water insoluble fraction is expressed in the fraction insoluble in hot sodium hydroxide, the fraction soluble in hot sodium hydroxide showing much less of an effect (Table III). There was no effect of 2,4-D on glucose incorporation into a lipid soluble fraction. The glucan fraction soluble in hot water, a fraction not included in data of Tables I and II, showed the greatest stimulation (30%) by 2,4-D added to the glucan synthetase assay.

TABLE III

SUBFRACTIONATION OF POLYSACCHARIDES OF THE *IN VITRO* ASSAY AND EFFECT OF 2,4-D ON INCORPORATION OF RADIOACTIVITY FROM GLUCOSE INTO VARIOUS SUBFRACTIONS

Onion stem explants were incubated 16-18 hours at 25° in the absence of 2,4-D. Plasma membrane fractions were isolated and glucan synthetase activity was assayed ± 5 μM 2,4-D added directly to the enzyme assay. Details of the polysaccharide fractionation are given in the text.

Auxin added to synthetase assay	Expt.	mμmoles glucose incorporated/hr/mg protein			
		Hot water	$CHCl_3$-MeOH	Hot NaOH	Hot NaOH Insoluble
None	II	85	21	104	280
	V	86	22	138	214
	VI	37	14	147	230
	Ave.	70	20	130	240
5 μM 2,4-D	II	123	19	126	317
	V	96	21	133	233
	VI	50	19	143	271
	Ave.	90	20	135	274
	Δ2,4-D, %	+30	0	+4	+14

TABLE IV

EFFECT OF MAGNESIUM IONS ON THE PLASMA MEMBRANE-ASSOCIATED GLUCAN SYNTHETASE AND THE *IN VITRO* RESPONSE TO 2,4-D

Plasma membrane fractions were isolated from stem explants preincubated in water. Units of specific activity are as described in Table III. The complete assay medium is 20 μM with respect to Mg^{++}. Average from 2 experiments.

Auxin added to synthetase assay	Synthetase assay medium	mμmoles glucose incorporated/hr/mg protein	
		Hot water soluble	Hot water insoluble
None	$-Mg^{++}$	61	88
	Complete	85	363
5 μM 2,4-D	$-Mg^{++}$	56	81
	Complete	110	404

The plasma membrane-associated glucan synthetase was markedly stimulated by magnesium ions (20 μM) and the 2,4-D-induced stimulation of activity was not observed in the absence of magnesium ions (Table IV). In the absence of magnesium ions, 2,4-D was without effect or inhibited the activity of the glucan synthetase. Again, the greatest stimulation by 2,4-D was shown by the hot water soluble fraction (30%) rather than the hot water insoluble fraction (11%).

A comparison of the relative effectiveness of *in vitro* and *in vivo* incubations with 2,4-D on the production of the hot water soluble glucan by isolated plasma membranes is given in Table V. In 3 experiments, a consistant but variable stimulation averaging 50% resulted from the inclusion of 2,4-D in the glucan synthetase assay with plasma membranes isolated from tissue incubated in the absence of 2,4-D. The inclusion of 2,4-D in the glucan synthetase assay of plasma membranes isolated from tissue incubated in the presence of 2,4-D had no additional effect except in Expt. II of Table V where incubation of the tissue with 2,4-D

TABLE V

COMPARISON OF IN VITRO AND IN VIVO STIMULATION BY 2,4-D OF THE SYNTHESIS OF HOT WATER SOLUBLE POLYSACCHARIDES BY THE PLASMA MEMBRANE-ASSOCIATED GLUCAN SYNTHETASE

Plasma membrane fractions were isolated from stem explants incubated as described in Table I. The unit of specific activity is mμmoles glucose incorporated into total hot water soluble polysaccharide/hr/mg protein.

Tissue incubation	Auxin added to Synthetase assay	Specific activity Expt. II	Expt. III	Expt. IV	Ave.
None	None	85	103	83	90
	5 μM 2,4-D (A)	123	111	169	134
	Δ 2,4-D, %	+45	+8	+103	+49
5 μ M 2,4-D	None	72	115	190	126
	5 μM 2,4-D (B)	129	112	185	142
	Δ 2,4-D %	+80	-3	-3	+12
Δ Tissue incubation with 2,4-D, %		-15	+39	+85	+40
	B/A	1.05	1.01	1.09	1.05

was not effective (-15% stimulation). In this experiment, addition of 2,4-D to the synthetase assay resulted in the apparent stimulation of 80% in the synthesis of hot water soluble glucans.

The interexperiment variability in the synthesis of hot water soluble glucans in response to 2,4-D in unexplained except on the basis of physiological differences among different plasma membrane preparations perhaps connected with endogenous auxin levels. Replicate assays rule out experimental error. Whatever the cause the differences are masked by 2,4-D present in the assay (Table V). When 2,4-D is added to plasma membranes from control tissue (A) and 2,4-D pre-incubated tissue (B) with the same experiment, the ratio of the specific activities of B/A are constant among experiments (Table V).

Discussion

A 50% enhancement of glucan synthetase activity by auxin pretreatment was previously reported for pea epicotyls by Abdul-Baki and Ray (1). The activity was localized within the Golgi apparatus of this tissue by Ray et al. (14). A similar enhancement, although not as great, appears to be associated with the plasma membrane-associated glucan synthetase of onion stem. Additionally, we show that 2,4-D added to the isolated plasma membrane fragments increases the specific activity of the glucan synthetase when the plasma membrane fragments are isolated from tissues which have not been pre-treated with auxin. Except for one experiment (Expt. II of Table V), 2,4-D added to plasma membranes isolated from tissues incubated with 2,4-D resulted in no additional stimulation. Mechanical analyses of frozen and thawed maize coleoptiles (7) suggested the possibility of an in vitro response of plant cell components to auxin. Our studies are the first direct biochemical demonstration of such a response. We find increased amounts of glucan synthetase resulting in vivo from pre-incubation of the tissue with 2,4-D (B/A ratios, Tables II and V). However, at least a portion of the in vivo response to 2,4-D may result from 2,4-D bound to the membrane and carried through the isolation procedure, i.e., a direct effect of the 2,4-D on the plasma membrane-associated synthetase.

Although the correspondence between wall deposition and cell elongation in growing tissues is not absolute, a consistent feature of both the in vivo experiments and the in vitro studies reported here is a stimulation of synthesis of polysaccharides soluble in hot water, a fraction specifically enhanced by auxin treatment of growing tissues (2,8,13). The physiological importance of such stimulations of polysaccharide synthesis remains unresolved. The still hypothetical enzymatic machinery which mediates auxin-induced cell wall changes may be unrelated to biosynthetic activities. Yet these enzymes may be structured as part of the plasma membrane (7). The

results are important in that a response to auxin has been obtained by adding the auxin to an *in vitro* system. They may indicate a more general response of enzyme systems of the plant plasma membrane to auxin. Independent studies by Villemez, *et al.* (C. L. Villemez, personal communication) show that a particulate glucan synthetase from mung beans, active in the synthesis of β-1,4-glucans (cellulose), is also stimulated by the *in vitro* addition of an auxin. In the studies of Villemez *et al.*, the auxin was indole-3-acetic acid (IAA).

Acknowledgments

We thank Dr. Lawrence Ordin, University of California, Riverside for helpful suggestions concerning the glucosyl synthetase assay procedure and Dr. C. L. Villemez, University of Ohio, Athens for access to unpublished information. The identification of the sodium hydroxide insoluble polysaccharides as β-1,3-linked glucans was provided by Dr. Ordin.

References

1. Abdul-Baki, A. A. and Ray, P. M., Plant Physiol. 47, 537 (1971).
2. Bonner, J., In Plant Growth Regulation (R. M. Klein, ed) Ames, Iowa State University Press, 1961, p. 307.
3. Feingold, D. S., Neufeld, E. F. and Hassid, W. Z., J. Biol. Chem. 233, 783 (1958).
4. Lembi, C. A., Morré, D. J., St.-Thompson, K. and Hertel, R., Planta 99, 37 (1971).
5. Morré, D. J., Plant Physiol. 45, 791 (1970).
6. Morré, D. J., Methods Enzymol. 22, 130 (1971).
7. Morré, D. J. and Eisinger, W. R., In Biochemistry and Physiology of Plant Growth Substances (F. Wightman and G. Setterfield, eds) Ottawa, Runge Press, 1968, p. 625.
8. Morré, D. J. and Olson, A. C., Can. J. Bot. 43, 1083 (1965).
9. Morré, D. J., Roland, J.-C., and Lembi, C. A., Proc. Ind. Acad. Sci. for 1969 79, 96 (1970).
10. Ordin, L. and Hall, M. A., Plant Physiol. 42, 205 (1967).
11. Ordin, L. and Hall, M. A., Plant Physiol. 43, 473 (1968).
12. Pinsky, A. and Ordin, L., Plant Cell Physiol., 10, 771 (1969).
13. Ray, P. M., Amer. J. Bot. 49, 928 (1962).
14. Ray, P. M., Shininger, T. L. and Ray, M. M., Proc. Nat. Acad. Sci. U.S. 64, 605 (1969).