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# Ultrastructural Alteration of Plant Plasma Membranes Induced by Auxin and Calcium Ions[1]

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## ABSTRACT

**Ultrastructural changes in isolated and *in situ* plasma membranes of etiolated soybean hypocotyls (*Glycine max* L. cv. Wayne) were induced by indole-3-acetic acid (IAA), other auxins, and calcium chloride. Fixed and embedded preparations were stained by a phosphotungstate-chromate procedure to identify and accentuate plasma membrane. Measurements were on micrographs obtained with an electron optical system calibrated and corrected for reproducible and accurate size measurements. Plasma membranes treated for 20 minutes with 1 μM IAA were 10 to 15% thinner than controls. The response to IAA was rapid, reproducible, auxin-specific, temperature-dependent, and reversible. Comparable responses were obtained with isolated and *in situ* membranes. Membranes treated with 0.5 M calcium chloride for 20 minutes were 15 to 20% thicker than controls. Multiple cycles of alternating calcium and IAA treatments yielded membranes with dimensions that reflected the last treatment of the series. The findings show a direct response of plasma membranes to growth regulating agents and provide evidence for a cell-free response of isolated plasma membranes to a hormone.**

The precise mode of action of plant hormones is unknown. This is true in particular for hormones of the auxin type such as IAA, and its synthetic counterpart, 2,4-D. One explanation of hormone action focuses on cell wall loosening as the mechanism of action (2), whereas other findings implicate effects on nucleic acid metabolism (7, 8, 11). A role for the plasma membrane in regulating cell wall properties is implicit from the physical closeness of the two structures. There is precedent for interaction of auxin hormones with the plasma membrane (4, 17). The latter findings (4) form a basis for integration of the seemingly disparate fast reactions of auxin action (1, 3, 12, 16) and the delayed transcriptional responses (7, 8, 11) into a postulated mechanism involving a single master reaction (15). This study provides evidence from high resolution electron microscopy which, in conjunction with fluorescence probe experiments of the accompanying paper (5), demonstrate that the plasma membrane exhibits a direct conformational response to auxin *in vitro* and *in situ*.

## MATERIALS AND METHODS

**Plant Material.** Soybean seeds (*Glycine max* L. cv. Wayne) were soaked in water overnight, planted in moist vermiculite, and germinated in the dark at 29 C in a constant temperature, high humidity chamber (4). Four days after planting, hypocotyls were excised and used for membrane isolations.

**Membrane Isolations.** About 10 g of hypocotyl tissue were homogenized at 0 to 4 C in 10 ml of one of the following media: medium A: 0.1 M K phosphate in freshly prepared and filtered coconut water and containing 20 mM EDTA and 0.5 M sucrose, final pH 7 (4, 17); medium B: deionized water, final pH of homogenate 5.8 ± 0.2; medium C: 0.5 M $CaCl_2$, pH 5.9 ± 0.1.

Hypocotyls were homogenized for 45 sec with a Polytron 20 ST (Kinematica, Lucerne, Switzerland) (9) operating at about 5,000 rpm. The homogenates were filtered through a single layer of Miracloth (Miracloth Sales, Chicopee Mills, N. Y.) to remove whole cells, tissue fragments, and cell walls. The filtered homogenates were divided among 8 to 10 lusterloid centrifuge tubes (5.4 ml) and centrifuged for 20 min at 10,000*g* (Sorvall RC2-B, HB-4 rotor, 8,000 rpm) in medium B or C to pellet membrane fragments. Since neither medium B nor medium C contained sucrose, it was possible to sediment most of the larger plasma membrane vesicles at 10,000*g*. With medium A, plasma membranes were purified by centrifugation on a discontinuous sucrose gradient (4). Details are provided in the accompanying paper (5).

**Membrane and Tissue Incubations.** All solutions were prepared in deionized water. With auxins, the final pH after the membranes were added was 6 ± 0.1. Except where indicated, the pH of the $CaCl_2$ solutions was adjusted to 5.9, but, except for data of Figure 6, the auxin solutions were unbuffered.

Incubations were initiated by rapidly resuspending the membrane pellets (using a Pasteur pipette) in 4.5 ml, or floating tissue pieces on 10 ml of the solutions to be tested (*e.g.* Table I). Incubations were terminated by addition of 0.5 ml of 20% glutaraldehyde in 1 M cacodylate, pH 7.2 ± 0.1, with rapid mixing, or by immersion of pellets or tissue in 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2 ± 0.1. Membranes were collected by centrifugation for 20 min at 10,000*g* (Sorvall RC2-B, HB-4 rotor, 8,000 rpm) and the resulting pellets were transferred to fresh fixative.

**Electron Microscopy.** Fractions and tissue were prepared for electron microscopy as described by Roland *et al.* (14). Sections were stained with a periodate-chromate-phosphotungstate procedure which accentuates and identifies plasma membranes. For comparison, sections were stained with lead citrate (13). Electron micrographs were obtained with a Philips EM200 that was calibrated and corrected for reproducible size measurements. Images were photographed on Kodak electron image plates at a magnification of 44,000. A 54,864-line diffraction grating replica (Ladd Research Industries) and negatively stained catalase crystals were used as magnification standards.

**Measurement of Membrane Thickness.** All measurements were on plasma membranes identified either from positional

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Table I. *Effect of Various Treatments and Pretreatments on Thickness of Plasma Membranes Isolated from Soybean Hypocotyls*

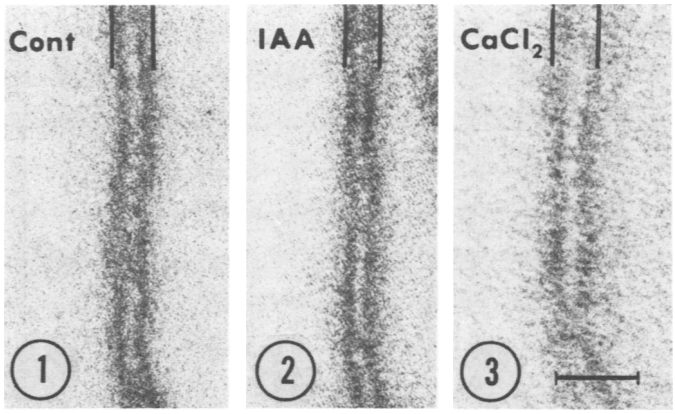
All treatments and pretreatments were for 20 min. Entries 1 to 23 were at 25 C. Entries 24 to 27 were at 0 C.

| Entry | Membranes Isolated In: | Pretreatment | Treatment | No. of Detns. | Membrane Thickness |
|---|---|---|---|---|---|
| | | | | | Å ± standard deviation |
| | | T = 25° | | | |
| 1 | $H_2O$ | None | None | 10 | 109 ± 4 |
| 2 | | | $H_2O$ | 20 | 105 ± 4 |
| 3 | | | 1 μM IAA | 10 | 93 ± 4 |
| 4 | | | 1 μM 2,4-D | 4 | 93 ± 6 |
| 5 | | | 1 μM NAA | 3 | 93 ± 4 |
| 6 | | | 1 μM Picloram | 3 | 97 ± 5 |
| 7 | | | 1 μM Benzoic Acid | 3 | 104 ± 2 |
| 8 | | | 1 μM 2,3-D | 3 | 103 ± 2 |
| 9 | | | 1 μM 2,5-D | 3 | 105 ± 2 |
| 10 | | | 0.5 M $CaCl_2$ | 12 | 118 ± 4 |
| 11 | | $H_2O$ | $H_2O$ | 4 | 103 ± 8 |
| 12 | | | 1 μM IAA | 3 | 91 ± 2 |
| 13 | | | 0.5 M $CaCl_2$ | 3 | 114 ± 2 |
| 14 | | 5 mM EDTA | $H_2O$ | 3 | 103 ± 1 |
| 15 | | | 1 μM IAA | 3 | 93 ± 2 |
| 16 | | | 0.5 M $CaCl_2$ | 3 | 102 ± 1 |
| 17 | $CaCl_2$ | None | None | 10 | 121 ± 6 |
| 18 | | | $H_2O$ | 7 | 107 ± 4 |
| 19 | | | 1 μM IAA | 10 | 88 ± 6 |
| 20 | CCM-EDTA | None | None | 10 | 105 ± 5 |
| 21 | | | $H_2O$ | 5 | 104 ± 5 |
| 22 | | | 1 μM IAA | 5 | 93 ± 5 |
| 23 | | | 0.5 M $CaCl_2$ | 6 | 107 ± 4 |
| | | T = 0° | | | |
| 24 | $H_2O$ | None | $H_2O$ | 4 | 107 ± 4 |
| 25 | | | 1 μM IAA | 4 | 105 ± 4 |
| 26 | $CaCl_2$ | None | $H_2O$ | 3 | 102 ± 1 |
| 27 | | | 1 μM IAA | 3 | 103 ± 3 |

relationships (in tissue) or by intense staining with the PACP[2] procedure (tissue and fractions). Images on negatives were magnified 12.5 times with a calibrated enlarging projector to give a final magnification of 550,000 × so that the measured dimensions were in the range of 4 to 7 mm. The final magnification was determined (±2.2%) from internal magnification standards. Measurements of the magnified images were made between the outer edges of the stained portions of the membrane profiles (Figs. 1–3) to the nearest 0.5 mm with a transparent ruler held perpendicular to the tangent of the vesicular profiles.

To avoid the difficulties inherent in making accurate size measurements of electron images, numerous potential sources of error were evaluated as part of a separate study. These included sampling methods, conditions of fixation and specimen preparation, time and temperature of PACP staining, support film thickness and carbon stabilization, section thickness, variation among and within vesicles, instrument variation, focus, processing and enlargement of negatives, and measurement procedures. The variation among replicate treatments was less than 2%. The variation among comparable treatments in different experiments was in the range of 2 to 5%.

For isolated membranes, at least four electron micrographs, each containing one to five randomly selected vesicle profiles of plasma membranes were measured. Three to 10 measurements were taken at random from each vesicle where the images were sharp and the vesicle membrane was nearly perpendicular to the plane of the section. Thus, a minimum of 10 to 15 measurements were obtained from each set of four micrographs. The measurements from the four micrographs were averaged to constitute one determination. For intact tissues, measurements were from eight to 10 different cells with 10 measurements/cell. All experiments were repeated at least three times. Each value is from a minimum of 120 total measurements from a total of 12 different electron micrographs representing three separate experiments. Deviations reported are standard deviations among the averages from all determinations for each of the three experiments.




FIGS. 1–3. Electron micrographs of plasma membrane vesicles stained with PACP and enlarged to a final magnification of 550,000 ×. The parallel lines illustrate the regions over which membrane thickness was measured. Membrane preparations were treated for 20 min at room temperature in 0.1 M sodium cacodylate buffer, pH 7, and then fixed for electron microscopy.

Fig. 1. Buffer alone.
Fig. 2. Buffer plus 1 μM IAA.
Fig. 3. Buffer plus 0.5 M $CaCl_2$.

## RESULTS

Plasma membranes in isolated preparations and in whole tissues from soybean hypocotyls stained by the PACP procedure

[2] Abbreviations: 2,3-D: 2,3-dichlorophenoxyacetic acid; 2,5-D: 2,5-dichlorophenoxyacetic acid; NAA: naphthaleneacetic acid; PACP: periodate-chromate-phosphotungstate.

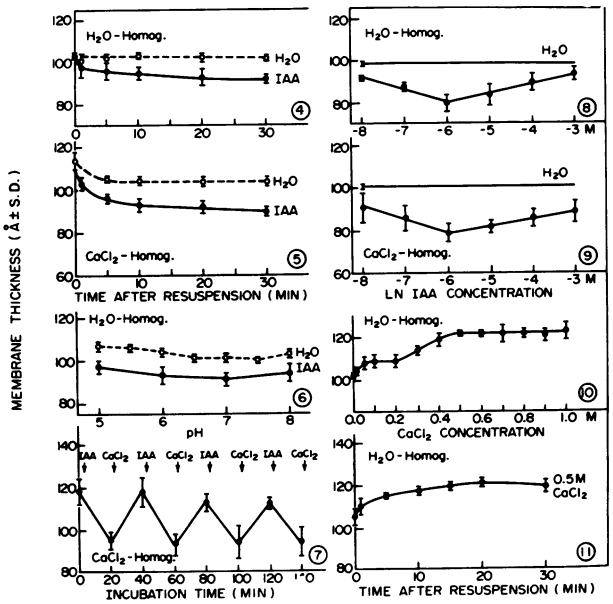
exhibited changes in thickness after treatment by IAA and $CaCl_2$ (Figs. 1–3; Table I). In all examples, numerical values represent the mean of the distribution of membrane thicknesses (see Fig. 12). Concentrations of $CaCl_2$ which inhibit elongation of hypocotyl segments caused the membrane profiles to thicken, whereas IAA at concentrations which promote elongation caused the membranes to appear thinner. The membrane thinning induced by auxins is accompanied by both a thinning of the light space in the center of the membrane and a thinning or loss of the material that stains with PACP (Figs. 1 and 2).

The response to IAA was rapid, with a half-time of about 1 min (Figs. 4 and 5), and was maximal within 20 min after IAA was added. Membrane thickness was effected very little by pH over the range of pH 5 to 8. The auxin responsiveness of the isolated membranes appeared independent of pH (Fig. 6). The dimensional changes induced by IAA and $CaCl_2$ were at least partially reversible, *i.e.* the membranes could be made alternately thick or thin by successive alternate treatments with $CaCl_2$ or IAA (Table I; Fig. 7). The final thickness of the membranes reflected the last treatment in a series. With successive cycles, recovery to previous thickness was not quite achieved, and there appeared to be a slight but progressive thinning of the membranes. The dose response relationships show the biphasic function characteristic of the growth response of stem tissues to auxins: an optimum at about 1 μM with an ascending limb (descending for growth) in the supraoptimal dose range, and a descending limb (ascending for growth) in the suboptimal dose range (Figs. 8 and 9). The IAA effect was reduced or eliminated at 0 C (entries 25 and 27, Table I). Several synthetic auxins (entries 4 to 6, Table I) such as NAA, 2,4-D, and picloram also caused membrane thinning, but benzoic acid and the growth-inactive analogs of 2,4-D, 2,3-D, and 2,5-D (entries 7 to 9, Table I) did not.

The response to $CaCl_2$ was maximal at 0.5 M (Fig. 10) with a 20-min incubation (Fig. 11), and was reduced or eliminated if the membranes were pretreated with 5 mM EDTA (entry 16, Table I) or if they were isolated in a medium containing EDTA (entry 23, Table I).

When treated with water, or $CaCl_2$, statistically normal distributions of membrane thicknesses were observed (Fig. 12). Membranes treated with water gave a broad distribution of thicknesses with a mean of about 100 A. Those treated with $CaCl_2$ were thicker and less broadly distributed with a mean of about 115 Å. The IAA-treated membranes were thinnest, and in two populations. The major population had a mean of about 90 Å. A second, minor population was also apparent with a mean of about 100 Å. These data, along with those of Table I and Figures 1 to 11, show a direct effect of a hormone on plasma membranes as evidenced by dimensional changes in a cell-free system.

The response of cell membranes of tissue explants *in situ* was similar to that of isolated membrane vesicles (Table II). In these experiments, the plasma membrane was identified by its position next to the cell wall so that membranes stained with either lead citrate or PACP could be compared. Measurements from sections of the same groups of cells showed that the dimensional changes were revealed only with the PACP stain in specimens treated with IAA, but that the $CaCl_2$-treated membranes appeared thicker with both lead and PACP-stained sections. In




FIGS. 4–11. Membrane thickness of isolated plasma membrane fragments. Except for data of Figure 6, solutions were unbuffered, pH 5.9 ± 0.1. Averages are from three experiments ± standard deviation.

FIG. 4. As a function of time, ± 1 μM IAA; homogenate prepared in water.

FIG. 5. As a function of time, ± 1 μM IAA; homogenate prepared in 0.5 M $CaCl_2$

FIG. 6. As a function of pH, ± 1 μM IAA in 0.1 M sodium cacodylate buffer; homogenate prepared in water.

FIG. 7. As a function of sequential incubation in either 1 μM IAA or 0.5 M $CaCl_2$ at intervals of 20 min each; homogenate prepared in 0.5 M $CaCl_2$.

FIG. 8. As a function of auxin concentration; homogenate prepared in water; ——, water control.

FIG. 9. As a function of auxin concentration; homogenate prepared in 0.5 M $CaCl_2$; ——, water control.

FIG. 10. As a function of $CaCl_2$ concentration; homogenate prepared in water.

FIG. 11. As a function of time after addition of 0.5 M $CaCl_2$; homogenate prepared in water.

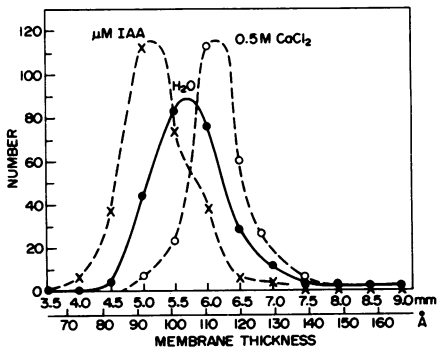



FIG. 12. Distribution of membrane thickness in Å and as measured in mm on enlarged images. The figure compares plasma membrane vesicles treated for 20 min with 1 μM IAA, $H_2O$, or 0.5 M $CaCl_2$. Each curve is from 250 measurements from five different experiments.

Table II. *Thickness of Plasma Membranes of Tissue Explants of Soybean Hypocotyl In Situ*

Treatments were with intact tissues floated on the solutions indicated for 1 hr after which the tissues were fixed and prepared for electron microscopy.

| Treatment (1 hr) | Staining procedure | |
|---|---|---|
| | Lead citrate | PACP |
| | Å ± standard deviation | |
| $H_2O$ | 87 ± 5 | 101 ± 3 |
| 1 μM IAA | 88 ± 6 | 87 ± 2 |
| 0.5 M $CaCl_2$ | 97 ± 5 | 115 ± 5 |

general, the membranes stained with PACP were about 30% thicker than those stained with lead, but with IAA-treated membranes, this difference was no longer evident. Comparisons of membrane thickness of endoplasmic reticulum, tonoplast, nuclear envelope, inner and outer mitochondrial membranes, and Golgi apparatus membranes in lead-stained sections revealed no differences due to auxin treatment (data not shown). Similar studies were not carried out with $CaCl_2$-treated cells.

## DISCUSSION

These experiments show a direct effect of a plant hormone on plasma membranes *in situ* and in a cell-free system. The response is reversible, specific, and dependent upon hormone concentration. We realize that the differences in membrane dimensions reflect differences in deposition of electron-opaque stain molecules. Yet, these staining differences represent differences inherent in the membranes. In the manner in which this study was carried out, they represent induced changes or transformations of the plasma membrane resulting from treatment with auxins or $CaCl_2$. Inolated plant membranes have been shown to bind auxins (6) and, in other studies (F. A. Williamson, K. Hess, and D. J. Morré, in preparation), the fractions that bind IAA were consistently enriched in plasma membranes.

The nature of the auxin-induced change remains problematic. The data in the accompanying report (5) on fluorescence polarization and lifetimes of a membrane-bound probe imply a small increase in microviscosity of local regions around the dye. These microviscosity changes could be a cause or a consequence of a more compact or condensed membrane resulting from auxin treatment. Data of Table II suggest that some component of the membrane, *i.e.* that portion which stains most intensely with the PACP procedure, is lost from the membrane. These observations are consistent with the findings of Hardin *et al.* (4) that some component having transcription factor activity is lost from isolated plasma membranes treated with auxins.

In contrast to IAA, the concentrations of $CaCl_2$ required for maximal effect on membrane thickening are not physiological. This could explain why Wheeler and Baker (18) did not observe thickness changes in membranes treated with 1 mM $CaCl_2$. Generally, membranes isolated in $CaCl_2$ exhibited a greater response to IAA than those isolated in water or medium A (Table I; compare Figs. 4 and 5 and Figs. 8 and 9). Therefore, $CaCl_2$ was included in the reversal experiments of Figure 7. It is clear that the membrane thickening induced by $CaCl_2$ occurs by a mechanism different from that of the IAA-induced membrane thinning, *i.e.* $CaCl_2$ is not functioning as an antiauxin. The $CaCl_2$ effect is abolished by EDTA treatment of the membranes but the IAA effect is not (Table I). Additionally, the $CaCl_2$ effect is expressed at 0 to 4 C, whereas the IAA response is expressed more slowly or not at all at these temperatures (Table I). The calcium responsiveness of the membranes appears related to some membrane component removed by EDTA and to membrane aggregation and fusion (10). Yet, for both the $CaCl_2$ and the IAA effect, it remains to be determined what the precise nature of the molecular changes involved is and whether the observed membrane response is a part of the functional growth response of plants to these growth-regulating agents.

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