Auxin and the Response of Pea Roots to Auxin Transport Inhibitors: Morphactin

Received for publication September 12, 1974 and in revised form January 22, 1975

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ABSTRACT

The auxin transport inhibitor methyl-1-2-chloro-9-hydroxyfluorene-9-carboxylic acid (CFM), a morphactin, inhibits negative geotropism, causes cellular swelling, and induces root hair formation in roots of intact Pisum sativum L. seedlings. In excised pea root tips, CFM inhibits elongation more than increase in fresh weight (swell ratio = 1.3 at 20 μM CFM). CFM growth inhibition was expressed in the presence of ethylene. Indoleacetic acid (IAA) prevented the expression of CFM growth inhibition possibly because IAA inhibited the accumulation of CFM into the tissue sections. CFM inhibited the accumulation of IAA and 2,4-dichlorophenoxyacetic acid into excised root tips. Applying Leopold's (1963, Brookhaven Symp. Biol. 16: 218-234) model for polar auxin transport, this result suggests a possible explanation for CFM inhibition of geotropism in pea roots, i.e. disruption of auxin transport by interfering with auxin binding.

Work with suspensions of intact cells (19) and work with particulate fractions from homogenates of shoot tissue (14-16, 23-25) indicates that some chemicals may inhibit auxin transport by interfering with auxin binding at auxin-specific receptor sites. CFM effects on geotropism, phototropism, apical dominance, and hypocotyl curvature correlate with the ability of this morphactin to inhibit auxin transport (6, 7, 18, 20, 21, 27). It occurred to me that a simple explanation for the CFM alteration of root geotropism might involve a mechanism whereby CFM interferes with auxin binding. The experiments reported in this paper indicate that this hypothesis may be true for pea roots.

MATERIALS AND METHODS

Plant Material. Seeds of Pisum sativum L. var. Alaska were imbibed for 6 hr, sown in vermiculite, and grown in the dark at 23 C.

Studies with Intact Seedlings. Two-day-old seedlings (roots 2-3 cm long) were soaked in water or 20 μM CFM for 5 min then placed horizontally in Petri dishes containing vermiculite moistened with the treatment solution. The experiment was then conducted in 10-liter desiccators as described elsewhere (8). Downward curvature of the root tips was measured at different time periods. After 24 hr, photomicrographs were made of freehand longitudinal sections taken from the elongation zones of the roots.

Growth Studies and Ethylene Evolution. Ten terminal 5-mm sections from 3-day-old seedlings (roots 5-6 cm long) were incubated in sealed 125-ml Erlenmeyer flasks as described previously (8) except that 6 ml of an incubation medium (pH 5.2) containing 5 mM dibasic potassium phosphate, 2 mM citric acid, 1 mM Ca(NO₃)₂·4H₂O, 1% sucrose (w/v), and appropriate concentrations of CFM or IAA were used. Ethylene evolution was assayed by gas chromatography using a flame ionization detector (1).

Studies with 14C-CFM and 14C Auxins. Tissue sections were incubated as described above, except that the buffer also contained 9-14C-CFM (10.4 mCi/mmmole, Cela Merck, Ingelheim, Germany), 14C-IAA (8.9 mCi/mmmole, New England Nuclear), or 1-14C-2,4-D (3.03 mCi/mmmole, Tracerlab). Radioactivity in the incubation medium was determined by counting an aliquot by liquid scintillation. After a given incubation period, radioactivity in the gas phase was determined by bubbling a 10-ml gas sample into 3 ml of NCS tissue solubilizer, then counting by liquid scintillation. The solution was then filtered, and the tissue was rinsed with ice water. After blotting and weighing, the sections were placed in a scintillation vial containing 2 ml of NCS tissue solubilizer. The vial was shaken at 45 C at least 4 hr. The radioactivity which had accumulated in the sections was then determined by liquid scintillation counting.

Metabolism of 14C-CFM was studied by homogenizing rinsed roots at 4 C in 5 ml of 50 mM potassium phosphate buffer, pH 6.8. The brei was extracted twice with 10 ml of chloroform. After evaporation of the chloroform under reduced pressure, the residue was dissolved in 50 μl of acetone. This residue and 14C-CFM were co-chromatographed separately with unlabeled CFM on an Eastman silica gel sheet. After development in benzene-glacial acetic acid (4:1, v/v), the chromatogram was cut into 1-cm strips. Each strip was placed directly into a vial for counting of the radioactivity by liquid scintillation.

RESULTS

CFM rapidly inhibited the normal geotropic response in roots of intact pea seedlings (Table 1; 20). By 24 hr, the elongation zone of the CFM-treated root showed swelling of the epidermal and cortical cells and many long root hairs (Fig. 1).

Dose-response data for growth of excised pea root tips (Fig. 2) showed that CFM inhibits elongation to a greater extent than increase in fresh weight. The increase in weight to increase in length ratio is 1.3 at 20 μM CFM (Fig. 2).

Application of auxin to pea roots increases ethylene pro-
duction (8, 9). Since CFM-induced growth inhibition was expressed (Fig. 2) in the presence of an ethylene concentration which is supraoptimal for growth inhibition (8), synergistic growth inhibition was expected in sections treated with CFM + IAA. Synergistic growth inhibition occurred only for length growth at 1 \( \mu \)M IAA and the two lowest CFM concentrations (Fig. 2). A mutual antagonism between all other CFM and IAA combinations seemed apparent for elongation (Fig. 2). IAA completely prevented CFM-induced fresh weight inhibition (Fig. 2).

The growth curve for CFM-treated roots was sigmoidal (Fig. 3); complete growth rate inhibition did not occur until after 12 hr. The pattern of IAA-induced growth inhibition (Fig. 3) was similar to that reported previously (9). In roots treated with CFM + IAA (Fig. 3), IAA controlled weight growth at all times and length growth for the first 12 hr. Between 16 and 24 hr, the elongation rate of roots treated with CFM + IAA exceeded the control elongation rate (Fig. 3). Thus, absolute elongation of roots subjected to the simultaneous treatment was greater than elongation of roots treated with CFM or IAA alone (Fig. 3). Figure 3 also shows that at no time did CFM increase ethylene production nor did CFM affect IAA-induced ethylene production.

The growth rate reduction caused by CFM after 12 hr (Fig. 3) might be due to tissue transformation of CFM into some other entity or possibly to slow tissue uptake of CFM. CFM remained unchanged during the 24-hr incubation period (Fig. 4). Presumably the initial rapid uptake of \(^{14}C\)-CFM (Fig. 5) represents uptake into the free space. \(^{14}C\)-CFM uptake began to level off after 1 hr (Fig. 5).

IAA had no effect on \(^{14}C\)-CFM metabolism (Fig. 4). After the first hour, accumulation of \(^{14}C\)-CFM in pea roots was inhibited by IAA (Fig. 5) concentrations as low as 0.1 \( \mu \)M (curve A, Fig. 6). This inhibition occurred in the presence of a saturating dose of unlabeled CFM (experiment B, Table II). In a 3-hr experiment, CFM also inhibited the accumulation of \(^{14}C\)-IAA in roots (Table III). Difficulties arise, however, in long term accumulation experiments using carboxyl-labeled IAA since much of the applied IAA is conjugated with aspartic acid or is decarboxylated (3, 9). 2,4-D remains intact in 24-hr experiments with pea roots (3); therefore, \(^{14}C\)-2,4-D was the radioactive auxin used for further accumulation studies. CFM concentrations as low as 0.01 \( \mu \)M inhibited accumulation of \(^{14}C\)-2,4-D in pea roots (curve B, Fig. 6). This inhibition occurred in the presence of a saturating dose of unlabeled 2,4-D (experiment A, Table II). Ethylene (1000 \( \mu \)l/l-0.1 \( \mu \)l/l), ABA (100 \( \mu \)M-1 \( \mu \)M), BA (10 \( \mu \)M), GA\(_3\) (100 \( \mu \)M), and the auxin transport inhibitors (5, 10) TIBA (50 \( \mu \)M-10 \( \mu \)M) and DPX1840 (10 \( \mu \)M) had no effect on \(^{14}C\)-CFM accumulation (data not shown). NPA, another auxin transport inhibitor, did inhibit \(^{14}C\)-CFM accumulation (Gaither and Abeles, in preparation).

Table I. Time Course for CFM Inhibition of Geotropism in Roots of Intact Pea Seedlings

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Degrees Negative Curvature (^{\circ})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>Control</td>
<td>7.8 ( \pm ) 2.7</td>
</tr>
<tr>
<td>CFM, 20 ( \mu )M</td>
<td>-3.5 ( \pm ) 1.6</td>
</tr>
</tbody>
</table>

\(^{1}\)Mean \pm confidence limit \((P < 5\%)\) from 24 seedlings per treatment.

Fig. 2. Effect of IAA and ethylene on CFM-induced inhibition of elongation and fresh weight increase in pea root tips. Incubation time was 24 hr. Controls grew 145% in length and 180% in fresh weight.
**Fig. 3.** Time course for CFM and IAA growth inhibition of and ethylene production from pea root tips. Vertical bars represent confidence limits ($P < 5\%$) from six replications.

**Fig. 4.** Chromatography of $^{14}$C-CFM and of radioactivity extracted from pea roots incubated for 24 hr with $^{14}$C-CFM plus or minus 10 $\mu$M IAA. $R_f$ of unlabeled CFM was 0.81.

**Fig. 5.** Effect of 10 $\mu$M IAA on the time course for $^{14}$C-CFM (0.13 $\mu$M) uptake by pea root tips. Each point is the mean of two replications.

**Fig. 6.** Unlabeled IAA inhibits accumulation of $^{14}$C-CFM and unlabeled CFM inhibits accumulation of $^{14}$C-2,4-D in pea root tips. Vertical bars represent ± SE of three replications. A: Roots were exposed for 22 hr to 0.13 $\mu$M $^{14}$C-CFM plus different concentrations of unlabeled IAA; B: roots were exposed for 4 hr to 0.41 $\mu$M $^{14}$C-2,4-D plus different concentrations of unlabeled CFM.

Accumulated radioactivity was lost from $^{14}$C-CFM-treated roots transferred from $^{14}$C-CFM solutions to buffer (Table IV). CFM-induced growth inhibition was also reversible (Table IV).

**DISCUSSION**

Leopold's (17) model of polar auxin transport suggests that auxin binds, actively moves across a membrane, then disengages from the transport site. If this model is accurate, then auxin movement could be inhibited by substances which are metabolic poisons and/or by substances which interfere with auxin attachment or disengagement from the transport site (17).
Table II. CFM Inhibits Accumulation of $^{14}$C-2,4-D (Experiment A) and IAA Inhibits Accumulation of $^{14}$C-CFM (Experiment B)

Pea root tips (three replications) were treated with the appropriate $^{14}$C compound plus saturating doses of the unlabeled chemicals, either singularly or in combination. Incubation times were (A) 16 hr and (B) 22 hr.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fresh Wt$^t$</th>
<th>$^{14}$C Accumulated In Roots$^t$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>% applied dpm</td>
</tr>
<tr>
<td>A. 0.41 μM $^{14}$C-2,4-D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>36.2 ± 0.7</td>
<td>11.79 ± 3.57</td>
</tr>
<tr>
<td>100 μM 2,4-D</td>
<td>34.5 ± 1.1</td>
<td>1.51 ± 0.22</td>
</tr>
<tr>
<td>100 μM CFM</td>
<td>41.3 ± 0.7</td>
<td>2.42 ± 0.34</td>
</tr>
<tr>
<td>100 μM 2,4-D + 100 μM CFM</td>
<td>32.9 ± 0.9</td>
<td>0.88 ± 0.13</td>
</tr>
<tr>
<td>B. 0.79 μM $^{14}$C-CFM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>56.7 ± 0.8</td>
<td>10.45 ± 1.46</td>
</tr>
<tr>
<td>100 μM CFM</td>
<td>49.1 ± 0.7</td>
<td>1.96 ± 0.09</td>
</tr>
<tr>
<td>100 μM IAA</td>
<td>29.6 ± 0.9</td>
<td>2.56 ± 0.30</td>
</tr>
<tr>
<td>IAA</td>
<td>29.6 ± 0.6</td>
<td>0.47 ± 0.01</td>
</tr>
</tbody>
</table>

1 Mean ± se.

2 Mean ± standard error (P < 5%).

I have not demonstrated a direct inhibition of auxin transport in pea roots by CFM. However, I observed, as others have (6, 20, 21, 27), that CFM inhibits root geotropism (Table I), a response thought dependent upon the root possessing a functional auxin transport system (22).

CFM does not inhibit respiration in several test systems (20, 21, 27); therefore, Leopold's (17) model predicts that CFM is not interfering with active auxin movement across a membrane. Thus, CFM may be interfering with auxin transport at the level of the auxin transport site. Indeed CFM did displace auxin from pea root tip sections (Fig. 6, Table III). Auxin also displaced CFM (Figs. 5 and 6).

To test the idea that an interference with binding might explain the CFM inhibition of auxin accumulation, experiment A of Table II was performed. I assumed that the presence of a saturating concentration (100 μM) of unlabeled 2,4-D would allow $^{14}$C-2,4-D to accumulate in the tissue only where a steady state exists between $^{14}$C-2,4-D and unlabeled 2,4-D, i.e. in tissue areas specific for the auxin. Unlabeled 2,4-D did inhibit $^{14}$C-2,4-D accumulation by 87%. In the presence of saturating doses of the auxin and CFM, $^{14}$C-2,4-D accumulation was reduced further by 6%. This figure represents CFM displacement of 2,4-D from tissue areas specific for the auxin. The tissue areas in question could be auxin binding sites.

Using a similar argument, auxin may be interfering with CFM binding. A saturating dose of unlabeled CFM displaced 81% of the $^{14}$C-CFM applied to excised tissue sections (experiment B, Table II). When a saturating dose of IAA was also present, $^{14}$C-CFM displacement was increased by 14% (experiment B, Table II).

It seems likely that the site of primary auxin action in roots is located at the cell wall or the plasma membrane (3, 22). Primary CFM action in pea roots may also occur at the cell surface. Two observations support this conclusion. First, accumulated CFM exists in an exchangeable, diffusible state which probably accounts for the reversibility of CFM-induced growth inhibition (Table IV). Second, CFM causes rapid inhibition of geotropism in intact roots (Table I) and rapid inhibition of growth in excised root tips (Fig. 3).

In time course studies, CFM showed two different effects on growth (Fig. 3). The first effect was a rapid inhibition from which the tissue was able to recover (Fig. 3). No growth rate recovery occurred from the second effect which was not expressed until after 12 hr (Fig. 3). This pattern of CFM-induced growth inhibition was not related to altered metabolism (Fig. 4) or uptake (Fig. 5) of CFM.

A suspected antagonism between CFM and IAA for elongation (Fig. 2) was not confirmed by time course studies (Fig. 3). Prior to 12 hr, elongation of sections treated with CFM + IAA was controlled by IAA-regulated processes, in this case probably ethylene production (Fig. 3) (8, 9). Ethylene has no effect on CFM accumulation (data not shown). IAA does not affect CFM metabolism (Fig. 4). Lack of expression of CFM-induced growth inhibition in the presence of IAA (Fig. 3) might be related to IAA reducing the level of CFM within the tissue (Figs. 5 and 6).

Tissue sections treated with CFM + IAA show an amplification of recovery from IAA-induced inhibition of elongation (Fig. 3). This response cannot be related to any known physiological process, but other workers (26) have reported a similar stimulation of root growth in seedlings which have been treated with auxin then transferred to an auxin-free growth medium. Aberg (2) has discussed this phenomenon of auxin-treated roots displaying positive after-effects.

Table III. Effect of CFM on $^{14}$C-IAA (0.14 μM) Accumulation and Destruction by Pea Root Tips

Means are based on three replications. Incubation time was 3 hr. In each column, means followed by the same letter are not significantly different (Student's t test).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fresh Wt$^t$</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>Solution$^t$</td>
</tr>
<tr>
<td>Control</td>
<td>32.6a</td>
<td>13.3b</td>
</tr>
<tr>
<td>CFM, 100 μM</td>
<td>32.3a</td>
<td>9.9c</td>
</tr>
</tbody>
</table>

1 Significant at the 0.1% level.

2 Significant at the 5% level.

Table IV. Time Course Relating Reversibility of CFM Elongation Inhibition to Reversibility of CFM Accumulation

For the elongation inhibition experiment, root tips were treated with 20 μM unlabeled CFM. For the accumulation experiment, root tips were treated with 0.13 μM $^{14}$C-CFM. Mean ± se based on three replications.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Elongation</th>
<th>$^{14}$C-CFM Accumulated In Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
<td>% applied dpm</td>
</tr>
<tr>
<td>Measured at 4 hr</td>
<td>1.10 ± 0.05</td>
<td>8.50 ± 0.30</td>
</tr>
<tr>
<td>Control</td>
<td>0.70 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>CFM</td>
<td>4.60 ± 0.20</td>
<td>12.93 ± 0.04</td>
</tr>
<tr>
<td>Measured at 16 hr</td>
<td>3.20 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.10 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>CFM</td>
<td>4.70 ± 0.18</td>
<td>13.75 ± 0.30</td>
</tr>
<tr>
<td>CFM 4 hr, then buffer 20 hr</td>
<td>6.20 ± 0.30</td>
<td>2.00 ± 0.10</td>
</tr>
<tr>
<td>CFM 16 hr, then buffer 8 hr</td>
<td>5.30 ± 0.25</td>
<td>6.25 ± 0.10</td>
</tr>
</tbody>
</table>

1 Buffer contained 7.10 ± 0.20% of applied dpm.

2 Buffer contained 6.00 ± 0.40% of applied dpm.
No increased ethylene production was detectable from excised root tips treated with CFM (Fig. 3). Thus, in this tissue, CFM or ethylene can separately cause cellular swelling, root hair induction (Fig. 1; refs. 1, 8), inhibition of geotropism (Table I; refs. 1, 8, 9), and inhibition of cell division (4, 27).

Polar auxin transport has been studied mainly in coleoptiles (10). From this work the concept has arisen that auxin transport and primary auxin action may have a common initial step (11, 13), namely auxin binding at auxin-specific receptor sites (12, 14). In pea roots, I have shown that CFM could be inhibiting auxin transport at the level of auxin binding; yet, CFM did not alter the pattern of fresh weight increase or ethylene production in the presence of auxin (Fig. 3). This result indicates that in this tissue, auxin transport may not be directly related to primary auxin action.

Acknowledgment—I thank Donald H. Lutz for performing superb technical assistance, Frederick B. Abeles for providing many salient ideas, R. C. French and R. A. Darrow for their interest in this work, J. P. Sterrett for help with liquid scintillation counting techniques, D. H. "Buster" Sauier, Jr. and A. T. McMannus for graphic art assistance, S. Gaither for emending the manuscript, Cela Meck, Ingelheim, Germany, for CFM, EMD-IT 3456, and 3H-CFM, Uni-Royal, Inc., for NPA, and C. W. Bingeman of E. I. du Pont de Nemours and Co., for DPX1840.

LITERATURE CITED