

Short-Term Treatment with Cell Wall Degrading Enzymes Increases the Activity of the Inositol Phospholipid Kinases and the Vanadate-Sensitive ATPase of Carrot Cells¹

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ABSTRACT

Treating carrot (*Daucus carota* L.) suspension culture cells with a mixture of cell wall degrading enzymes, Driselase, resulted in an increase in the percentage of [³H]phosphatidylinositol bisphosphate. Analysis of the lipid kinase activities in the isolated plasma membranes after whole cell treatment indicated that treatment with Driselase (2% weight/volume; the equivalent of 340 units per milliliter of hemicellulase and 400 units per milliliter of cellulase activity) or treatment with hemicellulase (31.7% weight/volume, 20.7 units per milliliter) resulted in an increase in the inositol phospholipid kinase activity. However, treatment with cellulase alone had no effect at 0.5% (weight/volume, 17.2 units per milliliter) or inhibited the kinase activity at 1% (weight/volume, 34.4 units per milliliter). The active stimulus in Driselase was heat sensitive. The plasma membrane vanadate-sensitive ATPase activity also increased when the cells were treated with Driselase. A time course study indicated that both the inositol phospholipid kinases and the plasma membrane vanadate-sensitive ATPase responded to as little as 5 seconds of treatment with 2% Driselase. However, at the lowest concentration of Driselase (0.04%, weight/volume) that resulted in an increase in inositol phospholipid kinase activity, the ATPase activity was not affected. Because inositol phospholipids have been shown to activate the vanadate-sensitive ATPase from plants (AR Memon, Q Chen, WF Boss [1989] *Biochem Biophys Res Commun* 162: 1295–1301), a stimulus-response pathway involving both the inositol phospholipid kinases and the plasma membrane vanadate-sensitive ATPase activity is discussed.

Cell wall degrading enzymes have been used routinely to isolate plant protoplasts since the first report by Cocking in 1960 (8). However, the stress incurred during cell wall digestion is severe and has been considered to be a major contributor to somaclonal variation typically found in plants regenerated from protoplasts. In this paper we address some of the earliest responses of plant cells to enzymatic cell wall digestion.

Our focus has been to study the changes in inositol phospholipid metabolism that result from treating carrot suspension culture cells with cell wall degrading enzymes. Since the first report that the polyphosphoinositides were present in

plants (4), there has been an interest in the role of these negatively charged lipids in plant signal transduction. Most of the components in the inositol phospholipid cycle in the signal transduction pathway found in animal cells have been found in plant cells. The polyphosphoinositides have been identified in suspension cultured cells (4, 9, 14, 37) and whole plant tissues (15, 28).

There are only a few reports, however, of rapid PIP² and PIP₂ metabolism in plants in response to external stimuli, and these may occur in selected systems (28). Many investigators have been unable to detect an increase in PIP₂-specific phospholipase C or in the production of inositol phosphates or DAG in response to external stimuli. When cultured parsley and soybean cells were challenged with elicitors, no change in the [³H]inositol labeled inositol phospholipids was found after 5 min (37). In studying plant growth regulators, Morr e *et al.* (27) found no significant change in DAG during auxin-stimulated growth of excised hypocotyl segments of soybean. Furthermore, treating isolated membranes of elongating soybean hypocotyls with the auxin, 2,4-D, caused a slight but insignificant change in the hydrolysis of [³H]PIP and [³H]PIP₂ (26, 34). An important consideration in all these studies is that PIP₂ levels in plant cells are about 0.05 to 0.5% of total inositol lipids (5, 9, 14) and IP₃ is rapidly metabolized in a cell free system (16, 25). Furthermore, there have been no reports showing that PIP₂ metabolism leads to an increase in cytosolic calcium or demonstrating the existence of a protein kinase regulated by DAG, calcium and phospholipid, *i.e.* protein kinase C (13).

To study signal transduction during cell wall digestion, we have treated carrot cells grown in suspension culture with cell wall degrading enzymes. Earlier studies on the effects of Driselase on the metabolism of [³H]inositol labeled polyphosphoinositides in fusogenic carrot cells showed that PIP₂ increased after 10 min of treatment and returned to the control level by 30 min (31). There was no detectable IP₂ or IP₃ either prior to or after the Driselase treatment. These data suggested that PIP₂ was not hydrolyzed by phospholipase C in response to Driselase treatment. We did not determine, however,

² Abbreviations: PIP, phosphatidylinositol-4-monophosphate; DAG, diacylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PIP₂, phosphatidylinositol-4,5-bisphosphate; LPI, lysophosphatidylinositol; LPIP, lysophosphatidylinositol-4-monophosphate; IP₂, inositol-1,4-bisphosphate; IP₃, inositol-1,4,5-trisphosphate.

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whether the increase in PIP₂ was caused by an increase in PIP kinase activity or a decrease in phospholipase activity. In this work, we have monitored the changes in the inositol phospholipids *in vivo* and the changes in inositol phospholipid kinase and vanadate-sensitive ATPase activities *in vitro* using isolated plasma membrane. *In vivo* treatment with Driselase caused a rapid increase (within 5 s) in the activity of the plasma membrane inositol phospholipid kinases and vanadate-sensitive ATPase.

Inositol phospholipids have been shown to directly affect membrane enzymes. For instance, PIP₂ has been shown to increase protein kinase C activity (6) and PIP and PIP₂ have been shown to increase the activity of Ca²⁺-ATPase of hepatocytes (20) and erythrocytes (7), the canine renal Na⁺/K⁺ ATPase (21) and the H⁺-ATPase of plant plasma membranes (24). In light of these data, a role for PIP and PIP₂ as direct effectors in signal transduction in plant cells is discussed.

MATERIALS AND METHODS

Plant Material

Wild carrot (*Daucus carota* L.) cells grown in suspension culture were transferred weekly as previously described (3) except that the macro salts of the fusion-inducing medium were made at half-strength by mixing them 1:1 (v/v) with the macro salts of the wild carrot medium. The cells were used on the fourth day after transfer.

Chemicals

Myo-[2-³H]inositol (20 Ci/mmol) and ³²Pi were purchased from New England Nuclear, [γ -³²P]ATP from ICN Radiochemicals, Driselase from Plenum Sciences, cellulase (lot No. 2601 chromatographically purified from *Trichoderma reesei*) from Worthington Biochemical Co., cellulysin from Calbiochem, and hemicellulase, pectinase, trypsin, and PIP were from Sigma Co. One unit of the cell wall degrading enzymes produced 1 μ mol of glucose or galactose per h. Vanadium oxide (gold label) was purchased from Aldrich Chemical Co., Inc., and a 5 mM stock solution was prepared according to Gallagher and Leonard (11).

Cell Harvest and Treatment

The cells were collected on filter paper (Whatman No. 1), rinsed with conditioned medium, and resuspended in 5 mL of conditioned medium in a 25 mL flask and placed on a shaker for 10 min at 110 rpm at a concentration of 0.1 g fresh weight/mL. For the 1 min treatment, aliquots of cells were transferred to test tubes, centrifuged at 2000g for 30 s and the supernatant was discarded. The treatments were added to the test tubes and the test tubes were shaken gently by hand. After 20 s, the cells were centrifuged for 30 s, the supernatant was discarded, and the reaction was stopped at 1 min by adding 3 mL of 0.4 osmolal sorbitol to the pelleted cells. For the 5 and 10 min treatments, the cells were treated in the same way as for the 1 min treatment except that the cells were incubated in 25 mL Erlenmeyer flasks at RT and shaken at 110 rpm.

Time Course Study

The cells were treated for different times with 2% (w/v) Driselase in 0.4 osmolal sorbitol, 2 mM Mes-KOH (2[N-

morpholino]ethanesulfonic acid) pH 4.8 or the 0.4 osmolal sorbitol-Mes buffer alone. At the end of the treatment, the reaction was stopped by diluting with the 0.4 osmolal sorbitol-Mes buffer to give a final concentration of 0.01% Driselase. The cells were quickly centrifuged at 2000g for 30 s. The supernatant was discarded and the cells were resuspended in 6 mL of the sorbitol-Mes buffer and pelleted once more; the supernatant was removed and the cells were homogenized and plasma membranes were isolated as described below. The time of treatment was reported as the time from the addition of Driselase or sorbitol until the cells were diluted with sorbitol. The total time after treatment until homogenization was less than 4 min.

Plasma Membrane Isolation

Plasma membranes were isolated as described by Wheeler and Boss (39) except that the cells were homogenized in a medium without lithium. Plasma membranes were suspended in 30 mM Tris/Mes buffer (pH 6.5) containing 15 mM MgCl₂ and 1 mM dithiothreitol. The test tubes were purged with nitrogen and the membranes were kept on ice until used for the lipid kinase or ATPase assays. Protein was determined by the method of Lowry with BSA as a standard (22).

Pi Uptake Assay

Carrot cells were labeled with 7.5 μ Ci/mL ³²Pi in 0.4 osmolal sorbitol (pH 4.8) and 1 μ M KH₂PO₄, 10 mM KCl for 5 min and then treated for 10 min with either sorbitol, Driselase, or boiled Driselase. At the end of the 10 min treatment, two aliquots of 100 μ L cells from each treatment were transferred to separate tubes containing 3 mL cold sorbitol and 3 mM KH₂PO₄. The cells were immediately centrifuged, the supernatant was discarded, and 3 mL cold sorbitol containing 3 mM KH₂PO₄ was added once more to rinse the cells. The final cell pellet from each tube was resuspended in 300 μ L of deionized water and 2 aliquots of 50 μ L each were counted in a liquid scintillation counter (Beckman LS 7000) after adding 5 mL Scintiverse II (Fisher).

Analysis of Lipid Kinases

Lipid kinase activity was measured in the presence of exogenous PIP (20 μ g) (36) unless mentioned in the figure legend. PIP was dissolved in CHCl₃:MeOH (2:1, v/v). Aliquots of the PIP solution containing 20 μ g of PIP were transferred to each test tube and dried under nitrogen. The plasma membranes (15–40 μ g protein) were resuspended in 50 μ L of buffer containing 0.2% Triton X-100 for 5 min according to Sandelius and Sommarin (33). For each experiment, equal amounts of membrane protein were transferred to the test tube containing PIP and preincubated for 5 min. When endogenous substrate was used, 0.01% Triton X-100 was added to enhance the uptake of ATP. Phosphorylation was started by adding 5 μ L ATP stock solution which contained 20 to 30 μ Ci[γ -³²P]ATP, 6.2 mM ATP, and 2.6 mM Na₂MoO₄ in 30 mM Tris/Mes and 15 mM MgCl₂ (pH 6.5), to give a final concentration of 0.56 mM ATP and 0.24 mM Na₂MoO₄. The tubes were shaken at 150 rpm for 15 to 20

min at room temperature, and the reaction was stopped by adding 1.5 mL ice cold $\text{CHCl}_3\text{:MeOH}$ (1:2, v:v). The lipids were kept on ice until they were extracted. The reaction has been shown to be linear for at least 20 min using isolated plasma membranes (36; JJ Wheeler, WF Boss, unpublished data).

Lipid Extraction and Separation

Lipids were extracted and separated by TLC as described by Boss and Massel (4) except that LK5D (Whatman) silica gel plates were presoaked in 1% potassium oxalate for 75 s, dried overnight at 100°C, and developed in $\text{CHCl}_3\text{:MeOH:NH}_4\text{OH:H}_2\text{O}$ (86:76:6:18, v:v:v:v). The distribution of the ^3H -labeled lipids was quantitated with a Bioscan System 500 Imaging Scanner. ^{32}P -Labeled lipids were quantitated as follows except where mentioned in the legends: an autoradiogram was made of each plate and the percentage represented by individual ^{32}P -labeled lipids was quantitated with a densitometer (Biomed Instruments) and multiplied by the total cpm per lane measured with the Bioscan Imaging Scanner. A similar ^{32}P distribution was found when individual spots were scraped from the plates and the ^{32}P distribution quantitated by liquid scintillation counting.

ATPase Assay

Vanadate-sensitive ATPase was assayed by adding 50 μL of the plasma membrane rich fraction (30–40 μg protein) to 350 μL reaction mixture to give a final concentration of 0.01% Triton X-100, 3 mM MgSO_4 , 30 mM Tris/Mes, 50 mM KCl, 1 mM NaN_3 in the presence or absence of 500 μM vanadate. The reaction was started by adding 100 μL of a 15 mM ATP stock solution and continued for 30 min at 200 rpm. The reaction was stopped by the addition of 250 μL 20% ice-cold TCA. Pi was determined according to the method of Taussky and Shorr (38).

RESULTS

Cell Wall Degrading Enzymes Affect [^3H]inositol Phospholipid Distribution

For the initial studies of the effects of cell wall degrading enzymes on phosphoinositide metabolism, carrot cells were labeled with [^3H]inositol for 3.5 h and treated for 10 min with 2% Driselase (w/v, in 0.4 osmolal sorbitol, the equivalent of approximately 400 units/mL of cellulase activity and 340 units/mL of hemicellulase activity). Because Driselase contains several types of cell wall degrading enzymes, *e.g.* cellulase, hemicellulase, pectinase, etc., it is very effective at removing plant cell walls to make protoplasts. At the light microscope level there was no evidence for the disruption of the cell wall after a 10 min treatment with Driselase. After about 2 h the cell wall is completely removed and over 90% of the cells are round protoplasts (3). In contrast, after a 2 h treatment with cellulase (1% w/v, 34.4 units/mL) or hemicellulase (31.7% w/v, 20.6 units/mL) alone, only about one or two protoplasts could be detected per 1,000 cells. Sorbitol (0.4 osmolal) is used to maintain the osmotic potential of the Driselase solution when making protoplasts, and thus for these studies, sorbitol was used as a control.

After 10 min of Driselase treatment two major changes in the inositol phospholipids were observed. LysoPIP decreased and PIP_2 increased (Fig. 1B). Boiled Driselase (2% w/v in 0.4 osmolal sorbitol) was used for comparison (Fig. 1A). When cells were labeled with [^3H]inositol for 18 h, a similar increase in [^3H]PIP₂ and decrease in lysoPIP was observed as a result of the Driselase treatment (Table I). A previous report indicated no detectable changes in IP₂ or IP₃ as a result of Driselase treatment (31). Boiled Driselase did not affect the inositol phospholipid distribution (Table I) suggesting that the stimulus was a heat-labile component of the Driselase. Cellulase (0.5%, w/v) alone caused a decrease in the percentage of [^3H] lysoPIP (Table I), but there was no significant increase in [^3H] PIP₂. Pectinase, cellulysin, and trypsin also had no significant effect on the percentage of [^3H]PIP₂ recovered from the labeled cells (data not shown).

Driselase may have acted as a stimulus or it may have produced carbohydrate fragments as a result of degrading the cell wall and these wall fragments may have acted as elicitors, *i.e.* stimuli. To determine if any cell wall fragments were responsible for the effects of Driselase on the inositol phospholipids, we obtained a wall fragment-enriched fraction. To do this, cells were treated for 10 min with Driselase; the cells were removed by centrifugation; the supernatant boiled for 20 min and centrifuged at 2000g to remove coagulated proteins and other precipitates. The supernatant was recovered as "boiled used Driselase" and was used to treat the [^3H] inositol-labeled cells. Boiled used Driselase had no effect on the [^3H]inositol phospholipid distribution (Table I).

In addition to the [^3H]inositol labeling studies, studies were done with cells prelabeled for 5 min with ^{32}P i. A comparison of the ^{32}P i incorporated during treatment with boiled and nonboiled Driselase indicated that after a 10 min treatment, the [^{32}P]PIP₂ was significantly greater in the cells treated with Driselase ($3,440 \pm 334$ cpm/g fresh weight, the mean of 6

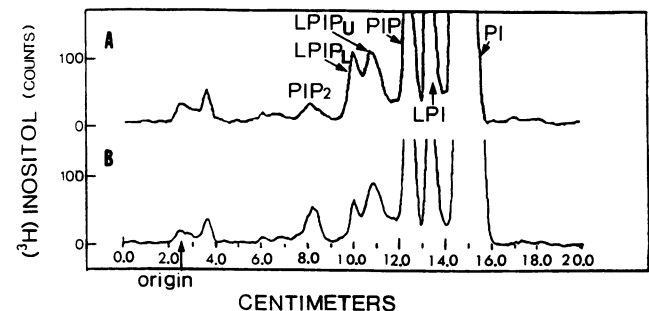


Figure 1. Effects of the cell wall digestion enzymes, Driselase, on [^3H]inositol-labeled phospholipids. Carrot cells were labeled with [^3H] inositol for 3.5 h and treated with 2% Driselase (B) or boiled Driselase (A) in 0.4 osmolal sorbitol in 2 mM Mes buffer (pH 4.8) for 10 min. Lipids were extracted, chromatographed on thin layer plates, and analyzed with a Bioscan System 500 Imaging Scanner. The profiles of the [^3H]inositol lipids from each treatment are shown. The scale has been expanded to show clearly the [^3H]PIP₂. The experiment was repeated at least four times and the trends were consistent. LPIP_u and LPIP_l are the two analogs of LPIP, *sn*-2, and *sn*-1 lyso-phosphatidylinositol-4-monophosphate, respectively.

values \pm SD) compared to that in the cells treated with boiled Driselase (2,365 \pm 459 cpm/g fresh weight). These data were consistent with the [³H]inositol labeling studies; however, Driselase and boiled Driselase both inhibited net Pi uptake relative to the sorbitol control (data not shown). Because of the effects of the heat-stable components in Driselase on ³²Pi uptake, the *in vivo* labeling studies were not continued.

The *in vivo* labeling studies indicated that Driselase caused a change in inositol phospholipid metabolism, but they did not delineate the mechanism involved. Several questions were left unanswered: Was the increase in the PIP₂ caused by an increase in PIP kinase activity or inhibition of PIP₂ hydrolysis? How rapidly did the cells respond to Driselase? To answer these questions, plasma membrane vesicles were isolated from treated and control cells and the lipid kinase activities were monitored.

Treating Cells with Cell Wall Degrading Enzymes Increases Plasma Membrane Inositol Phospholipid Kinase Activities

Phosphorylation of the plasma membrane lipids was studied using [γ -³²P]ATP (Fig. 2). As shown by Sommarin and Sandelius (36), plant plasma membranes have very active DAG and PI kinases and detectable PIP kinase activity producing phosphatidic acid (PA), PIP, and PIP₂, respectively. The two ³²P-labeled compounds migrating between PA and PIP are probably lysoPA and PA pyrophosphate. These ³²P-labeled lipids were not studied further.

Treating cells with Driselase for 10 min resulted in a preferential increase in [³²P]PIP and [³²P]PIP₂ produced in the phosphorylation assay using isolated plasma membranes (Fig. 2). For the experiment shown in Figure 2 there was a threefold increase in [³²P]PIP₂, and a 30% increase in [³²P]PIP, and an 11% increase in [³²P]PA as a result of the Driselase treatment. The same amount of membrane protein was used for each assay. In all instances the total recovered ³²P/mg protein was greater from the Driselase/sorbitol-treated cells than from those treated with sorbitol alone. The increase in [³²P]PIP₂ also was seen with endogenous substrate (data not shown); however, because PIP₂ formation was low with these membranes and because changes in PIP kinase activity were the focus of this study, all subsequent experiments were done with exogenous PIP added as a substrate. The relative increase in [³²P]PA after Driselase treatment varied daily as is evident

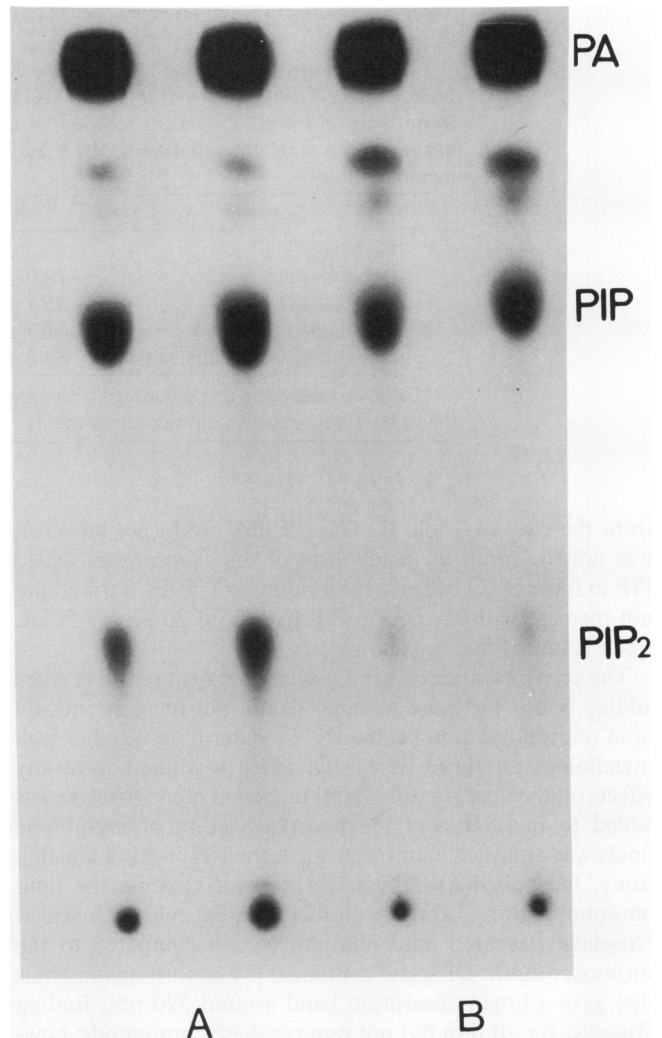


Figure 2. An autoradiogram showing the effects of *in vivo* treatment with Driselase on plasma membrane inositol phospholipid kinase activities. The cells were treated for 10 min with 2% Driselase in 0.4 osmolal sorbitol with 2 mM Mes buffer (pH 4.8) (A) or in the sorbitol buffer alone (B). The plasma membranes were isolated as described in "Materials and Methods" and 15 μ g membrane protein plus 20 μ g PIP were used per assay. Standards migrated as noted.

Table I. Changes in the Distribution of Inositol Phospholipids during Cell Wall Digestion

The cells were labeled with [³H]inositol (0.2 μ Ci/mL) for 18 h and treated for 10 min with 2% (w/v) Driselase, 2% (w/v) boiled Driselase, 2% (w/v) boiled used Driselase, or 0.5% (w/v) cellulase. All treatments were in 0.4 osmolal sorbitol (pH 4.8); therefore, sorbitol was used as the control. The data are presented as percentages of the total [³H]inositol-labeled lipids. Data are the average of two numbers. The experiment has been repeated at least six times, and the trends are consistent.

	Sorbitol	Boiled Driselase	Driselase	Boiled Used Driselase	Cellulase
	% total [³ H]inositol-labeled lipids				
PI	88.0 \pm 0.38	86.61 \pm 0.25	89.38 \pm 0.81	87.24 \pm 0.15	91.35 \pm 0.20
PIP	3.28 \pm 0.37	4.18 \pm 0.45	4.33 \pm 0.14	4.33 \pm 0.04	3.74 \pm 0.07
LPIP	7.31 \pm 0.71	7.24 \pm 0.35	4.59 \pm 0.70	7.06 \pm 0.09	3.82 \pm 0.17
PIP ₂	0.25 \pm 0.01	0.30 \pm 0.05	0.58 \pm 0.15	0.20 \pm 0.02	0.30 \pm 0.07
cpm/g fresh wt	11,095 \pm 3,335	9,670 \pm 3,930	6,855 \pm 2,705	11,725 \pm 1,225	10,735 \pm 885

Table II. Effects of *in Vivo* Treatment with Driselase on the Phosphorylation of Plasma Membrane Lipids

Carrot cells were treated for 10 min with either Driselase, boiled Driselase, boiled used Driselase, or boiled Driselase pretreated with charcoal in 0.4 osmolal sorbitol containing 2 mM Mes (pH 4.8) or sorbitol alone. Plasma membranes were isolated as described in "Materials and Methods." Representative values for the sorbitol control were: PA 432,200 ± 4,133; PIP 117,500 ± 3,900; PIP₂ 18,724 ± 197 (cpm/mg protein).

Treatment	PA ^a	PIP	PIP ₂
Sorbitol	100	100	100
Driselase	178.9 ± 62.6	213.9 ± 91.6	333.4 ± 70.7
Boiled Driselase	35.1 ± 5.3	40.5 ± 6.9	52.7 ± 3.7
Boiled used Driselase	50.7 ± 4.0	56.4 ± 41.4	60.5 ± 19.5
Boiled Driselase + charcoal	59.8 ± 16.8	59.2 ± 19.4	88.0 ± 8.7

^a Data are presented as percentage of cpm relative to that in the sorbitol control plus/minus standard deviation of four values from two experiments.

from the data in Table II. The [³²P]PA produced, however, was not the result of metabolism of the exogenously added PIP to form DAG because the amount of [³²P]PA formed did not increase with increasing PIP from 5 to 20 μg per 50 μL assay volume (data not shown).

The active components in Driselase are heat sensitive since adding boiled Driselase to the cells did not increase inositol lipid phosphorylation (Table II). To determine whether wall metabolites produced during Driselase treatment have any effects on the lipid phosphorylation, boiled used Driselase was added to the cells and the phosphorylation of membrane lipids was analyzed. Consistent with the [³H]inositol labeling study, the boiled used Driselase did not increase the lipid phosphorylation (Table II). In fact, treating cells with boiled Driselase decreased lipid phosphorylation compared to the sorbitol controls. Driselase contained UV absorbing materials that gave a broad absorption band around 320 nm. Boiling Driselase for 20 min did not remove these compounds; however, adding charcoal to the Driselase removed the UV absorbing compounds and decolorized the solution (data not shown). The charcoal treatment also decreased the effect of boiled Driselase on [³²P]PIP₂ production (Table II). These data suggested that charcoal-binding compounds, such as phenolics, may decrease the lipid phosphorylation.

Driselase treatment *in vivo* did not affect the activity of nonspecific membrane phosphatases or the degradation of the inositol phospholipids in the *in vitro* assay. When molybdate, a nonspecific phosphatase inhibitor, was added to the reaction mixture it had little effect on the phosphorylation of the lipids from membranes isolated from either Driselase-treated or control cells (data not shown). Furthermore, chase experiments with 3.5 mM nonradioactive ATP also indicated no detectable changes in the decrease of ³²P-labeled lipids over a 10 min period when comparing the membranes from Driselase and sorbitol treated cells. These data are in agreement with those of Sandelius and Sommarin (33) and of Einspahr *et al.* (10), who showed little inositol phospholipid degradation in isolated plant plasma membranes.

The Driselase effect was not due to residual Driselase that had coisolated with the membranes because if Driselase was added directly to the membranes, all lipid phosphorylation decreased (Table III). The decreased phosphorylation when Driselase was added to the membranes appeared not to be caused by phosphatases in Driselase since there was no detectable phosphatase activity using *o*-carboxyphenyl phosphate as a substrate (data not shown). In addition, molybdate was present in the reaction mixture to inhibit nonspecific phosphatases. Furthermore, if Driselase along with 3.5 mM

Table III. Effects of Driselase Added Directly to the Plasma Membrane on Inositol Phospholipid Kinase Activity

Cells were harvested and the plasma membranes were isolated as described in "Materials and Methods." Plasma membranes (25 μg) were treated for 2 min and 20 s with 50 μL of either 0.5% (w/v) cellulase, 2% (w/v) Driselase pretreated with charcoal, 2% (w/v) Driselase, 4% (w/v) Driselase in H₂O, or H₂O alone, at which time the phosphorylation was started by adding 10 μL of 40 μCi [γ-³²P]ATP.

	H ₂ O	Driselase ^a (4% w/v)	Driselase (2% w/v)	Driselase + Charcoal (2% w/v)	Cellulase (0.5% w/v)
PA	100	22.5 ± 3.1	38.5 ± 1.1	49.6 ± 3.4	86.0 ± 13.2
PIP	100	24.8 ± 3.9	36.5 ± 2.0	65.1 ± 17.7	85.0 ± 18.8
PIP ₂	100	23.2 ± 2.1	37.8 ± 0.6	41.0 ± 5.3	66.1 ± 7.0
Total counts	100	25.1 ± 3.0	39.9 ± 1.1	51.1 ± 3.7	80.9 ± 10.9

^a Data are presented as changes in the [³²P]phospholipid in the treatments relative to that in water control plus/minus standard deviation of four values from two experiments.

nonradioactive ATP was added to the membranes for 5 min immediately following the 20 min phosphorylation with [γ - 32 P]ATP, there was no effect on the 32 P-labeled lipid distribution or on the amount of 32 P-lipid recovered (data not shown). These data indicated that the decrease in lipid phosphorylation as a result of adding Driselase directly to the membrane was not the result of increased lipid catabolism. Removing the UV absorbing compounds by pretreating the Driselase with charcoal partly overcame the inhibition seen when Driselase was added directly to the membranes (Table III). This is consistent with the effects of *in vivo* treatment with these compounds on lipid phosphorylation (Table II).

In an attempt to determine which enzymes in Driselase were causing the increase in lipid kinase activity, several commercially available cell wall degrading enzymes were used. Cellulase (0.5% w/v; 17.2 units/mL; lot No. 2601) had no effect on the inositol lipid phosphorylation and a slight inhibition of [32 P]PA formation when added to the cells for 10 min (Table IV). At twice the concentration (1.0% w/v), cellulase treatment of the cells actually decreased all lipid phosphorylation. Hemicellulase (31.7% w/v; 20.7 units/mL), however, stimulated the lipid kinase activity. Neither cellulase nor hemicellulase alone were effective at releasing protoplasts so that the effect of these enzymes did not result from a difference in the physical denuding of the cell; and yet, there were distinct differences in the ability of the two enzymes to elicit a response with regard to the plasma membrane lipid kinase activity.

Response to *in Vivo* Treatment with Cell Wall Degrading Enzymes Seen in 5 s

The response to Driselase is rapid. After the cells were exposed to Driselase for 5 s, the plasma membrane PI and PIP kinase activities increased compared to DAG kinase activity (Fig. 3). The increase in the percentage of [32 P]PIP reached a plateau after 5 s but that of [32 P]PIP₂ had a tendency to increase compared to the sorbitol control. When cells were treated with sorbitol alone as a control, the percentage of PA, PIP, and PIP₂ decreased at 5 s but returned to approximately the values at time zero by 10 s. While the absolute changes in the kinase activity varied daily, these transient changes always were observed in the control membranes at the 5 s time point.

Cell Wall Degrading Enzymes Also Cause an Increase in Plasma Membrane ATPase Activity

The plant plasma membrane H⁺-ATPase is an E1-E2-type ATPase, and, like the Na⁺/K⁺-ATPase found in animal cells, it is inhibited by vanadate (11). The vanadate-sensitive ATPase activity of the carrot cell plasma membrane fraction increased almost threefold as a result of 10 min *in vivo* treatment with Driselase (Table V). If 2% Driselase was used, the response was seen after as few as 5 s of treatment (Fig. 4). At this high concentration of Driselase, the time course of the response of the lipid kinases and ATPase could not be separated. When lower concentrations of Driselase were tested, it was evident that the lipid kinase activity was more sensitive to Driselase than the vanadate-sensitive ATPase activity was (Fig. 5). After 1 min treatment with 0.04% (w/v) Driselase, PIP kinase activity increased while the vanadate-sensitive ATPase activity did not increase significantly.

Vanadate Increases PIP Kinase Activity

Vanadate has been shown to increase the activity of PI kinase from *Torpedo californica* (17). Vanadate also increased the activities of the carrot inositol phospholipid kinases (Fig. 6). Adding 250 μ M vanadate during the plasma membrane isolation resulted in increased kinase activity in the control, sorbitol-treated, cells both with endogenous and exogenously added PIP (data not shown). With membranes from Driselase-treated cells, vanadate only increased phosphorylation with the endogenous substrate (Fig. 6, A-C). When exogenous substrate was added, no further increase beyond that found with Driselase treatment was evident (Fig. 6, D-F). The effect of vanadate was not on the stimulus *per se* since adding 300 μ M vanadate to Driselase during the *in vivo* treatment did not enhance the effects of Driselase on the inositol lipid kinase activity (data not shown).

DISCUSSION

Rapid changes were detected in the activities of plasma membrane enzymes as a result of treating cells with cell wall degrading enzymes. *In vivo* studies using [3 H]inositol-labeled

Table IV. Effects of *in Vivo* Treatment with Cellulase or Hemicellulase on the Phosphorylation of Inositol Phospholipids

Cells were treated for 10 min with 0.4 osmolal sorbitol (pH 4.8), cellulase 0.5% (w/v) or 1% (w/v), or hemicellulase 31.7% (w/v) in the sorbitol solution. Plasma membranes were isolated as described in "Materials and Methods" and 15 μ g membrane protein plus 20 μ g PIP were used for the phosphorylation. 32 P-labeled lipids were quantitated with a Bioscan System 500 Imaging Scanner.

	Sorbitol	Cellulase ^a 17.2 units/mL (0.5% w/v)	Cellulase 34.4 units/ mL (1% w/v)	Hemicellulase 20.7 units/mL (31.7% w/v)
PA	100	54.0 \pm 14.3	22.5 \pm 3.5	185.1 \pm 102.4
PIP	100	110.5 \pm 25.8	45.0 \pm 13.0	185.5 \pm 69.9
PIP ₂	100	120.8 \pm 24.8	66.5 \pm 12.9	170.8 \pm 32.5

^a Data are presented as relative changes in the phosphorylation of each lipid compared to that in the sorbitol control plus/minus standard deviation of at least six numbers from three experiments.

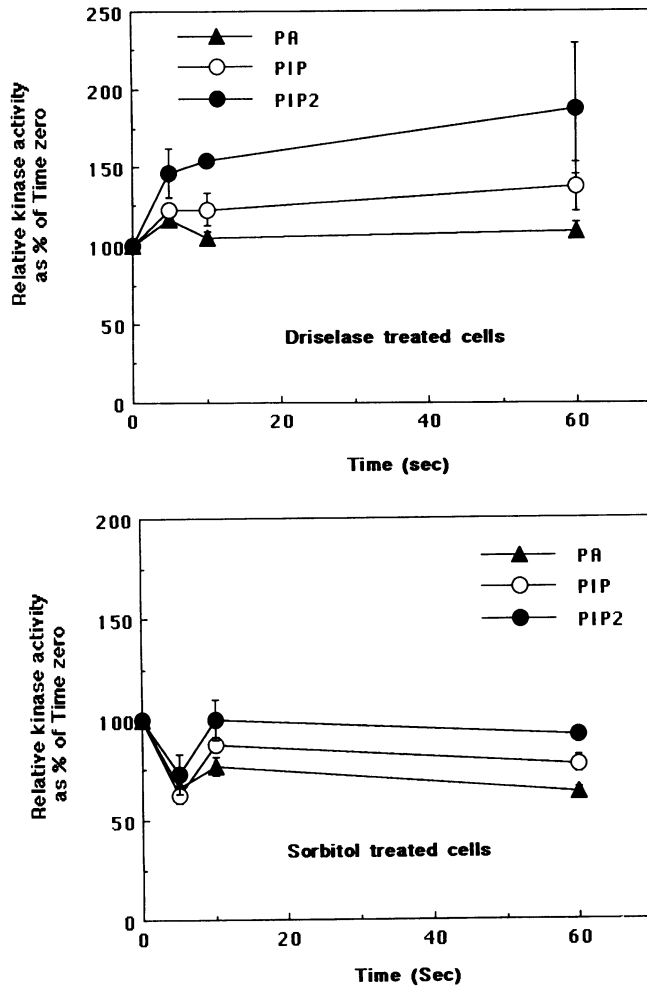


Figure 3. Changes in the plasma membrane inositol phospholipid kinase activities in response to *in vivo* Driselase treatment over time. Data are presented as changes in the percentage of each lipid from each treatment relative to those in the untreated control at time zero. Each number is an average of four replicates from two experiment. Error bars show the standard deviation. The total cpm in the Driselase-treated samples were at least four times higher than those of the control (11,950 cpm). The cpm for each phospholipids in the control were: PA, 2,613; PIP, 1,578; and PIP₂, 720. Plasma membranes were isolated as described in "Materials and Methods" and 15 μg membrane protein plus 20 μg PIP were used per assay.

cells indicated that Driselase caused an increase in PIP₂ and a decrease in lysoPIP (this work) and no detectable increase in IP₂ or IP₃ (31). ³²Pi labeling *in vivo* and lipid kinase assays *in vitro* indicated that PIP₂ synthesis had increased compared to that in the control cells. Both Driselase and hemicellulase treatment of the cells increased [³²P]PIP₂ formation in the *in vitro* phosphorylation assay. In contrast, cellulase treatment either had no effect or decreased the formation of PIP₂ depending on the concentration of cellulase used.

The increase in PIP₂ formed was not the result of increased substrate availability since the increase in lipid phosphorylation was evident when exogenous PIP as well as when endogenous substrate was used. Vanadate also increased the phosphorylation of inositol phospholipids with endogenous sub-

Table V. Effects of Driselase on the Vanadate-Sensitive ATPase Activity of Carrot Plasma Membranes

Cells were treated for 10 min with either 0.4% osmolal sorbitol (pH 4.8) or 2% (w/v) Driselase in sorbitol or conditioned medium. Plasma membranes were isolated as described in "Materials and Methods" and 30 μg membrane protein was used for each ATPase assay. Due to daily variation in the ATPase activity the representative values given are the average of two numbers from one experiment. The mean of four numbers from two experiments plus/minus standard deviation are given as percent of control.

Treatment	Pi	Control
	$\text{nmol mg}^{-1} \text{ protein min}^{-1}$	%
Conditioned medium	49.3 ± 5.0	100
Sorbitol	58.6 ± 5.4	118.3 ± 12.8
Driselase	127.0 ± 5.5	259.3 ± 8.5

strate or exogenous substrate when plasma membranes from sorbitol-treated cells were used but only with endogenous substrate when Driselase-treated cells were used. The effect of vanadate on the inositol phospholipid kinase activity suggests either the presence of a phosphorylated regulatory protein or a vanadate-induced change in the enzyme or membrane that affected the V_{max} of the kinases (17).

The stimulation of the plasma membrane enzymes is seen only if the treatments are given *in vivo*. The fact that adding Driselase or cellulase directly to the isolated membranes did not increase kinase activity suggested that these enzymes indirectly affected the inositol phospholipid kinase activities and that some component(s) of the cytoskeleton, cytoplasm, or cell wall was necessary for the response. One potential mechanism is that upon stimulation of the cells, a soluble kinase became bound to the plasma membrane thus increasing the specific activity of the enzyme in the isolated membrane fraction. There are both soluble and membrane-associated PI kinases in the carrot cells (W Gross, CM Okpodu,

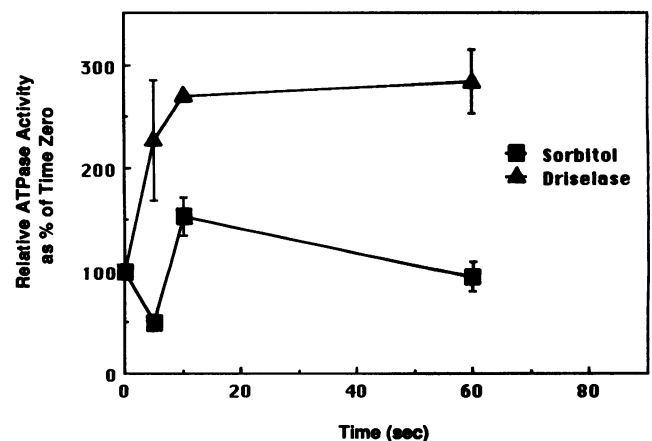


Figure 4. Changes in the plasma membrane vanadate-sensitive ATPase in response to *in vivo* Driselase treatment over time. Data are presented as the percentage of the activity in the treatments compared to that in the untreated controls at zero time. Each number is an average of four replicates from two experiments. Error bars show the standard deviation. The ATPase activity in the control is $59.1 \pm 9.6 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$.

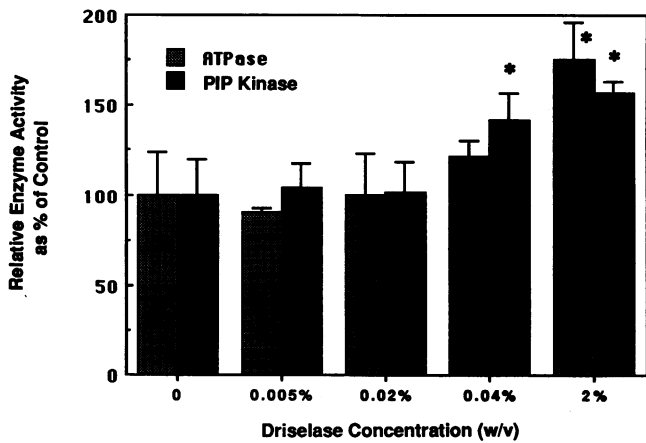


Figure 5. Changes in the activity of plasma membrane vanadate-sensitive ATPase and PIP kinase in response to different concentrations of Driselase. The cells were treated for 1 min with varying concentrations of Driselase. Data for the PIP kinase and ATPase activities are presented as described in Figures 3 and 4, respectively, except that they are relative to the 1 min sorbitol control. The total cpm in the sorbitol control was 23,038 and the cpm in each phospholipid of that treatment were: PA, 5,182; PIP, 4,054; LysoPIP, 1,759; and PIP₂, 1,914. The ATPase activity in the sorbitol control is 66.6 ± 16.0 nmol·min⁻¹·mg⁻¹ protein. The plasma membranes were isolated as described in "Materials and Methods" and 25 μg membrane protein was used per assay. The asterisks denote values statistically different from the control. The experiment has been repeated twice and the trends were consistent.

WF Boss, unpublished results). A similar scenario has been described for protein kinase C where the amount of protein kinase C bound to the membrane increases when cells are stimulated (18).

Another possible mechanism for Driselase to activate the inositol phospholipid kinases indirectly is through production of elicitors. When carrot cells were exposed to the cell wall degrading enzymes, the cell wall would be hydrolyzed producing potential elicitors (12, 19). Cell-free extracts of elicitors have been reported to induce many responses similar to the respective pathogens (1, 30, 32). While it is possible that the stimulus was a cell wall component released by the enzyme, the boiled supernatant after 10 min of Driselase treatment, which should have contained wall fragments, did not stimulate the plasma membrane enzymes. We also have treated cells with boiled enzyme extracts from cells treated only 5 min with the enzymes. For these experiments, the extracts were separated on Sephadex G25 and then boiled to denature the enzymes. None of the potential elicitor fractions increased lipid kinase activity. These data do not completely rule out wall fragments, however, because the fragments may have precipitated with the protein upon boiling or they may not have been able to penetrate to the membrane without the active enzyme.

Neither hemicellulase nor cellulase alone were effective at removing the cell wall at the concentrations used, and yet, hemicellulase, like Driselase, increased lipid phosphorylation and cellulase decreased lipid phosphorylation. Thus, the effects of the cell wall degrading enzymes on the inositol phospholipid kinases did not seem to result from physically altering the cell wall environment.

The decrease in lipid phosphorylation when Driselase is added directly to the membrane appeared to be partially caused by compounds that bound to charcoal. Charcoal pretreatment of Driselase partially eliminated the effect indicating that some nonenzymatic, possibly phenolic compounds, in the Driselase might have decreased DAG and inositol phospholipid kinase activities. Phenolic analogues have been

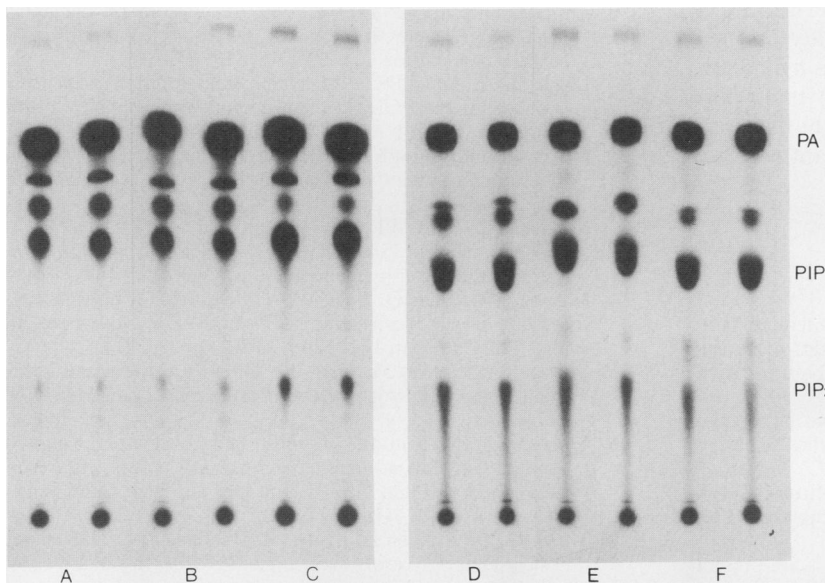


Figure 6. An autoradiogram showing the effects of vanadate on inositol phospholipid kinase activities of the plasma membranes from Driselase-treated cells. The cells were treated with Driselase for 10 min and the plasma membrane was isolated as described in "Materials and Methods" (lanes A, D) except that 1 mM NaOH (lanes B, E) or 250 μM V₂O₅ in 1 mM NaOH (lanes C, F) was included in the isolation medium and in phosphorylation medium. In all instances the pH was adjusted to that of the nontreated controls. The experiment has been repeated twice and the trends were consistent. Lipid phosphorylation was done in the absence (lanes A, B, C) or presence (D, E, F) of exogenous PIP (20 μg).

shown to decrease the [³²P]PIP formed by A431 cell membranes (29). Driselase also was found to decrease protein phosphorylation when added directly to membranes and to selectively activate calcium-dependent protein phosphorylation when added *in vivo* to carrot cells (2).

Analysis of the effects of Driselase on the plasma membrane ATPase showed that like inositol phospholipid kinases, the plasma membrane vanadate-sensitive ATPase activity increased when cells were treated with 2% Driselase. However, at a low concentration (0.04%, w/v) of Driselase, only inositol phospholipid kinases were affected. Based on these data and the fact that inositol phospholipids, but not PA, will increase vanadate-sensitive ATPase activity when added to isolated plasma membrane (24), we propose the following sequence of events for signal transduction in the carrot cells. First, the inositol phospholipid kinases are activated. Second, the increase in inositol phospholipid leads to activation of the membrane ATPase. Because the plasma membrane vanadate-sensitive ATPase in the oat root cells may be regulated by a Ca²⁺-stimulated kinase (35), an effect of Driselase on this or other kinases can not be ruled out.

In summary, we have shown that the activities of the plasma membrane inositol phospholipid kinases and the vanadate-sensitive ATPase increase rapidly after treating cells with cell wall degrading enzymes. The fact that IP₂ and IP₃ were not detectable even in stimulated carrot cells is consistent with the low levels of PIP₂ found in higher plants (5, 9, 14). In addition, there are no reports of a DAG-activated, calcium, and phospholipid-dependent protein kinase in plant cells (13). These data suggest that PIP and PIP₂ may be involved in regulation of cellular metabolism without being hydrolyzed by phospholipase C. PIP and PIP₂ have been shown to enhance the plasma membrane Ca²⁺-ATPase of hepatocytes (20) and erythrocytes (7), the Na⁺/K⁺-ATPase from canine renal tissue (21), and the vanadate-sensitive ATPase of plants (24). In addition PIP₂ has been shown to increase protein kinase C activity (6).

Our working hypothesis is that the negatively charged inositol phospholipids directly affect membrane enzyme activity in plants and that one mechanism for plants to respond to external stimuli and change their pattern of growth is to affect the inositol phospholipid kinases and thereby affect plasma membrane ATPase activity. Consistent with the hypothesis are the observations of Memon and Boss (23) that when etiolated sunflower hypocotyls are exposed to light there is a decrease in lipid kinase activity and a concomitant decrease in ATPase activity.

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