Changes in Phosphatidylinositol Metabolism in Response to Hyperosmotic Stress in *Daucus carota* L. Cells Grown in Suspension Culture

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Carrot (*Daucus carota* L.) cells plasmolyzed within 30 s after adding sorbitol to increase the osmotic strength of the medium from 0.2 to 0.4 or 0.6 osmolar. However, there was no significant change in the polyphosphorylated inositol phospholipids or inositol phosphates or in inositol phospholipid metabolism within 30 s of imposing the hyperosmotic stress. Maximum changes in phosphatidylinositol 4-monophosphate (PIP) metabolism were detected at 5 min, at which time the cells appeared to adjust to the change in osmoticum. There was a 30% decrease in [3H]inositol-labeled PIP. The specific activity of enzymes involved in the metabolism of the inositol phospholipids also changed. The plasma membrane phosphatidylinositol (PI) kinase decreased 50% and PIP-phospholipase C (PIP-PLC) increased 60% compared with the control values after 5 min of hyperosmotic stress. The PIP-PLC activity recovered to control levels by 10 min; however, the PI kinase activity remained below the control value, suggesting that the cells had reached a new steady state with regard to PIP biosynthesis. If cells were pretreated with okadaic acid, the protein phosphatase 1 and 2A inhibitor, the differences in enzyme activity resulting from the hyperosmotic stress were no longer evident, suggesting that an okadaic acid-sensitive phosphatase was activated in response to hyperosmotic stress. Our work suggests that, in this system, PIP is involved in the initial response to hyperosmotic stress but may be involved in the recovery phase.

The signal transduction mechanisms involving inositol phospholipid-derived second messengers, IP$_3$ and diacylglycerol, are well characterized in many animal cells (Majerus et al., 1986; Berridge and Irvine, 1989; Michell, 1992). In these systems the response includes a rapid and transient decrease in PIP$_2$ and at least a 3-fold increase in IP$_3$. The increased IP$_3$ releases calcium from internal stores, specifically the ER (Streb et al., 1983), thus activating calcium-dependent enzymes and altering cell physiology.

Most components of the inositol phospholipid-mediated signal transduction pathway have been shown to be present in higher plants (for reviews, see Einspahr and Thompson, 1990; Rincón and Boss, 1990; Chen et al., 1991; Gross and Boss, 1992; Hetherington and Dröbak, 1992). However, only in the *Samanea saman* pulvini is there evidence for stimulus-mediated turnover of polyphosphorylated inositol phospholipids and inositol phosphates within the rapid time scale seen in animal cells (Morse et al., 1987). Although results from in vitro studies and from IP$_3$ microinjection in vivo have shown that IP$_3$ can release Ca$^{2+}$ from internal stores such as vacuoles and tonoplast vesicles (Dröbak and Ferguson, 1985; Schumaker and Sze, 1987; Ranjeva et al., 1988; Gilroy et al., 1990), the complete pathway from a stimulus to Ca$^{2+}$ release has yet to be demonstrated in higher plants.

When studying the functions of the inositol phospholipids, one needs to realize the multifaceted effects of the inositol phospholipids on cell physiology. For example, in addition to being the sources of second messengers, PIP and PIP$_2$ also can directly affect cell physiology by regulating P-type ATPases (Varsanyi et al., 1983; Schäfer et al., 1987; Momen et al., 1989a; Chen and Boss, 1991), protein kinase C (Chauhan and Brockerhoff, 1988; Chauhan et al., 1989), and actin polymerization and nucleation sites (Lassing and Lindberg, 1985; Forscher, 1989). It has been shown that both PI and PIP kinase are present in the F-actin fractions from both plant (Tan and Boss, 1992) and animal cells (A431 mouse fibroblast cells) (Payrastre et al., 1991) and that the specific activities of the lipid kinases in the F-actin fraction change in response to external stimuli. These data suggest that there is a change in intracellular enzyme distribution or covalent modification of the enzymes. In addition, we recently (Yang et al., 1993) have found a protein that not only regulates PI kinase activity but also binds and bundles actin and has translational elongation factor-1a activity. Thus, changes in the metabolism of these very negatively charged phospholipids could be a part of a complex intracellular communication system among the plasma membrane, cytoskeleton, and protein synthesis machinery.

Changes in inositol phospholipids had been shown to occur in *Dunaliella salina* as a result of hyperosmotic stress (Ein-
spahr et al., 1988), and changes in inositol phosphates have been reported for beet roots exposed to different osmoticum (Srivastava et al., 1989). Because cells grown in suspension culture provide a relatively uniform system for biochemical studies, we decided to study the effects of hyperosmotic stress on inositol lipid metabolism in this system in more detail. For these experiments we kept the cells in their normal medium and applied stress by adding sorbitol to the medium rather than placing the cells in sorbitol alone.

Plasmolysis has been used over the years by cell biologists to study the properties of the plasma membrane of plant cells (Pfeffer, 1877; Lee-Stadelmann and Stadelmann, 1976). Plasmolysis of cells in response to hyperosmotic conditions is rapid (within seconds) and readily visible with the light microscope. Rapid membrane depolarization occurs as the cells plasmolyze (Slayman, 1982); however, the mechanism of signal transduction has not been elucidated. Tolerance to osmotic stress is important agriculturally. Recent advances in this field show promising results in altering cell physiology and applied stress by adding sorbitol to the medium rather than placing the cells in sorbitol alone.

The question we have addressed in this work is what role do the inositol phospholipids play in cellular responses to hyperosmotic stress? Our data indicate that the inositol phospholipids are involved in the recovery phase as the cells adjust to hyperosmotic conditions and that this response may be mediated by an okadaic acid-sensitive phosphatase.

**MATERIALS AND METHODS**

**Plant Material**

Wild carrot (Daucus carota L.) cells grown in suspension culture were transferred weekly and used 4 d after transfer as previously described (Chen and Boss, 1990).

**Chemicals**

myo-[2-3H]inositol (24.40 Ci mmol⁻¹) and PI-4-P (inositol-2-3H[N]) (9.90 Ci mmol⁻¹) were purchased from New England Nuclear, and [γ-32P]ATP was purchased from ICN Radiochemicals. 3H-Labeled inositol phosphate standards (I-1-P, I-4-P, IP2, I-1,3,4-P3, IP3, I-1,3,4,5-tetrakisphosphate, I-hexaphosphate) were obtained from New England Nuclear. [14C]I(1,3,4)P3 was prepared from [14C]inositol-labeled parotid acinar cells (Hughes et al., 1989). Dowex-1 (X8), chloride form (dry mesh, 200–400), and okadaic acid were purchased from Sigma, and okadaic acid stock (155 μM) was prepared in 10% DMSO according to manufacturer’s guide. All other chemicals were analytical grade.

**Hyperosmotic Treatment and Okadaic Acid Pretreatment**

The cells from several 125-mL culture flasks were combined into a 500-mL flask and redistributed by pipetting 22.5 mL of medium and cells into 125-mL flasks. The flasks were placed on a shaker for 30 min at 120 rpm and 25°C to preequililibrate the cells. The 30-min equilibration period is essential to stabilize the cells and minimize the effects of transfer. The conditioned culture medium had an osmolality of 0.2 osm, determined by freezing point depression using an Osmette Precision Osmometer and a pH of 4.2. Hyperosmotic conditions were produced by adding 2.5 mL of 2 or 4 osm sorbitol in conditioned medium containing 2 mM Mes buffer (pH 4.2) to conditioned medium (22.5 mL, pH 4.2). This gave a final osmoticum equivalent to 0.4 and 0.6 osm, respectively. An equal volume of conditioned medium containing 2 mM Mes buffer (pH 4.2) was added for the control. At 30 s before the termination of the treatments or at time zero for the 30-s time points, the cells were quickly poured into conical tubes and centrifuged at 2000g for 30 s. Supernatant was poured off, and cells were immediately placed on ice and homogenized or extracted for inositol phospholipid or inositol phosphate analysis.

Cells were pretreated with okadaic acid for 1 h following the 30-min preequilibration. Okadaic acid was added using a stock solution of 155 μM okadaic acid in 10% (v/v) DMSO to give a final concentration of 0.15 μM okadaic acid and 0.01% DMSO. The equivalent volume of 10% DMSO was added as a control.

**Observations of Plasmolysis**

For the microscopy studies, the carrot suspension culture cells (0.1 g in 4.5 mL of conditioned medium, pH 4.2) were placed in a small Erlenmeyer flask (25 mL) and treated hyperosmotically by adding 0.5 mL of 2 or 4 osm sorbitol in conditioned medium containing 2 mM Mes buffer (pH 4.2), to give a final osmolality of 0.4 and 0.6 osm, respectively. A drop of cells was placed on the glass slide, and plasmolysis was observed over time using a Zeiss IM 35 microscope with a ×63 neofluor lens. The first observations were made 50 to 40 s after the addition of osmoticum.

**[3H]Inositol Labeling**

Cells (approximately 0.25 g fresh wt 25 mL⁻¹ of medium) were used 3 d after transfer. myo-[2-3H]inositol (0.2 μCi mL⁻¹ for lipid analysis and 0.4 μCi mL⁻¹ for inositol phosphate analysis) was added to the cells for 18 h. The cells were harvested by brief centrifugation (2000g for 30 s).

**Extraction and Separation of Inositol Phosphates in HPLC**

The centrifuged cells were quenched with a final concentration of 4% (v/v) perchloric acid (Kirk et al., 1990) for inositol phosphate analysis. Perchlorate-quenched cell extracts were neutralized with freon/ctylylamine (Kirk et al., 1990), and the inositol phosphates therein were resolved on an Adsorbosphere 5 μ-SAX HPLC column (Alltech Associates, Deerfield, IL) using a slight modification of the salt gradient previously described (Menniti et al., 1990) generated from water and buffer (1 mM ammonium dihydrogen phosphate, pH 3.35 with phosphoric acid): 0 to 10 min, 0% buffer; 10 to 85 min the buffer increased linearly from 0 to 35%; 85 to 130 min, the buffer increased linearly from 35 to 80%. Half-minute fractions were collected for 80 min, and 1-min fractions were collected thereafter. Scintillant was added to each of the fractions, and the radioactivity was assessed.
Lipid Extraction and Separation

For lipid analysis 1.5 mL of ice-cold CHCl₃:MeOH (1:2, v/v) was added to the centrifuged cells, and the lipids were extracted and separated by TLC as described by Cho et al. (1991). Briefly, the LKSD (Whatman) silica gel plates were presoaked in 1% potassium oxalate for 80 s, dried in a microwave for 10 min, and developed in CHCl₃:MeOH: NH₄OH:H₂O (86:7:6:16, v/v/v/v). The distribution of the ³²P-labeled phospholipids was visualized with a Bioscan System 500 imaging scanner. The efficiency was 1% for ³H and 10% for ³²P. ³²P-Labeled phospholipids were visualized by autoradiography.

Plasma Membrane Isolation

Plasma membranes were isolated by aqueous two-phase partitioning as described by Chen and Boss (1990). The final plasma membrane pellet was resuspended in 30 mM Tris/Mes buffer (pH 6.5) containing 15 mM MgCl₂ and kept on ice until used for the enzyme assays. For the PIP-PLC assay, plasma membranes were resuspended in 50 mM Tris-HCl, pH 6.0. Protein was determined by the method of Lowry et al. (1951) with BSA as a standard.

Analysis of PI Kinase

PI kinase activity was measured in the absence and presence of exogenous substrate. Equal amounts of plasma membrane protein were used for the control and treated cells within each experiment. The protein amount ranged from 15 to 40 µg for the experiments reported. The plasma membranes were resuspended in 40 µL of 30 mM Tris/Mes buffer (pH 6.5) containing 15 mM MgCl₂. Phosphorylation was started by adding 10 µL of ATP stock solution, which contained 10 µCi of [γ-³²P]ATP (7000 Ci mmol⁻¹), 6 mM ATP, 0.05% Triton X-100, and 5 mM Na₂MoO₄ in 30 mM Tris/Mes containing 15 mM MgCl₂ (pH 6.5), to give a final concentration of 1.2 mM ATP, 1 mM Na₂MoO₄, and 0.01% Triton X-100. When exogenous substrate (PI, 25 µg/tube) was added, the final concentration of Triton X-100 was 0.2% and the pH was 8.0. The PI was presolubilized in 2% Triton X-100. The tubes were shaken by hand vigorously every 1 min at room temperature, and after 10 min, the reaction was stopped by adding 1.5 mL of ice-cold CHCl₃:MeOH (1:2, v/v). The lipids were kept on ice until they were extracted.

ATPase Assay

Vanadate-sensitive ATPase was assayed by adding 50 µL of the plasma membrane-rich fraction (30–40 µg of protein) to 350 µL of reaction mixture to give a final concentration of 0.01% Triton X-100, 3 mM MgSO₄, 30 mM Tris/Mes, 50 mM KCl, 1 mM NaN₃ in the presence or absence of 500 µM vanadate as described by Gallagher and Leonard (1982). The reaction was started by adding 100 µL of a 15 mM ATP stock solution and continued for 30 min at room temperature. The reaction was stopped by the addition of 250 µL of 20% ice-cold TCA. The Pi released was determined according to the method of Taussky and Shorr (1953).

PLC Assay

PLC activity was assayed according to the method of Melin et al. (1992). The standard reaction mixture contained 50 mM Tris-HCl (pH 6.0), 20 µM Ca²⁺ (a Ca²⁺/EGTA mixture was used as described by Owen [1976]), 0.2 mM PIP, and 4 to 6 µg of membrane protein in a final volume of 50 µL. The reaction was started by the addition of a PIP stock solution containing ³²P-labeled PIP (1100 dpm nmol⁻¹) in 0.1% deoxycholate, which was prepared by drying the lipid under a stream of nitrogen followed by sonication in 0.1% deoxycholate for 10 min. The reaction was stopped after 5 min at 25°C by addition of 1 mL of chloroform:methanol (2:1, v/v). After addition of 250 µL of 1 n HCl, vortexing, and brief centrifugation in a table-top centrifuge, the radioactive reaction products were recovered in the upper phase, analyzed by ion-exchange chromatography on a Dowex AG 1-X8 column (Berridge et al., 1983), and quantitated by liquid scintillation counting.

RESULTS

Hyperosmotic Stress Induces Rapid Plasmolysis

The osmolality of the medium in which the carrot cells were growing was determined to be approximately 0.2 osm. Cells in conditioned medium were turgid and contained large starch granules (Fig. 1A). When sorbitol was added to the medium to give the equivalent of a 0.4 osm solution, the outer cells in a cluster could be seen to plasmolyze slightly (Fig. 1B). At a final concentration of 0.6 osm, plasmolysis was complete within 10 min.
clearly evident and complete in all cells within 30 s. The photograph in Figure 1C was taken after 45 s. The plasmolyzed cells could be seen to regain volume slightly over a 5-min period (Fig. 1D). After 5 to 10 min the volume stabilized. The average decrease in diameter of the cells in 0.6 osm sorbitol was 15 ± 4.8% (n = 6).

The Distribution of [3H]inositol-Labeled Lipids Changes after Hyperosmotic Stress

In the [3H]inositol-labeled carrot cells the predominant [3H]inositol-labeled lipid was PI (82–85% of the total, Table I). [3H]PIP and [3H]PIP2 were relatively minor constituents (approximately 8 and 0.5%, respectively). When carrot cells were treated hyperosmotically with sorbitol in conditioned medium to give an osmoticum of 0.4 or 0.6 OSM, the total amount of [3H]-labeled lipid did not change significantly at either 30 s or 5 min (Table I). Furthermore, within the time frame that plasmolysis was complete (30 s), there were no significant changes in any of the [3H]inositol-labeled lipids based either on percentage of total [3H]-labeled lipid or on cpm (Table I).

After the 5-min treatment at either 0.4 or 0.6 OSM sorbitol, the proportion of [3H]PIP in the [3H]-labeled lipids decreased by 25 and 32%, respectively, relative to the controls (Table I, P < 0.05). This effect on PIP, which appears to be a secondary response to osmotic stress, was not associated with any significant change in the levels of [3H]PIP2. Because we only detected changes in [3H]PIP, we focused our studies on PIP metabolism.

The changes in [3H]PIP in response to osmotic stress could have resulted from stress-induced changes in the kinase/phosphatase cycle that interconverts PI and PIP. Osmotic stress also might have caused an increased rate of hydrolysis of PIP by PLC. One potential mechanism by which such putative regulation could be achieved is by covalent modification of the enzymes or of regulatory proteins. Therefore, we isolated the plasma membranes of carrot cells and assayed PI kinase activity and PIP hydrolysis by PLC as well as the plasma membrane vanadate-sensitive ATPase activity, specifically to search for any stable changes in the inherent activities of these enzymes.

Hyperosmotic Stress Decreases the Plasma Membrane Vanadate-Sensitive ATPase Activity

Initially as a means of characterizing the plasma membrane fractions, we studied the vanadate-sensitive ATPase activity in plasma membranes isolated from stressed and nonstressed cells. Greater than 85% of the ATPase activity in the plasma membrane fraction from the carrot cells was inhibited by vanadate (data not shown). The specific activity of the vanadate-sensitive ATPase decreased when cells were treated hyperosmotically at 0.4 OSM by adding sorbitol to the conditioned medium (Fig. 2). A reduction in the ATPase-specific activity was detected within 1 min. The maximum decrease was observed after 5 min of hyperosmotic stress.

Hyperosmotic Stress Increases the Activity of PIP-PLC

Plasma membranes prepared from the stressed cells exhibited a transient increase in PIP hydrolysis by PLC. No effect was observed after 2 min of stress. A 60 ± 6.4% stimulation in activity was observed after 5 min, and by 10 min the rate of hydrolysis of PIP had returned to the control level (Fig. 3). Analysis of the water-soluble products by Dowex-1 anion-exchange chromatography showed that more than 90% were

Table I. Changes in the distribution of [3H]inositol lipids in response to 0.4 and 0.6 OSM hyperosmotic stress for 30 s and 5 min

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]inositol-Labeled Lipids</th>
<th>PI</th>
<th>Lyso-PI</th>
<th>PIP</th>
<th>Lyso-PIP</th>
<th>PIP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>83.0 ± 2.7*</td>
<td>2.5 ± 0.7</td>
<td>8.1 ± 0.9</td>
<td>5.3 ± 2.0</td>
<td>0.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>(7496 ± 54)b</td>
<td>173 ± 6</td>
<td>(673 ± 45)</td>
<td>(324 ± 23)</td>
<td>(43 ± 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 OSM</td>
<td>83.1 ± 2.8</td>
<td>2.7 ± 0.7</td>
<td>7.5 ± 1.2</td>
<td>5.2 ± 2.1</td>
<td>0.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>(7464 ± 89)</td>
<td>204 ± 40</td>
<td>(676 ± 74)</td>
<td>(302 ± 18)</td>
<td>(45 ± 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6 OSM</td>
<td>82.3 ± 2.7</td>
<td>3.2 ± 1.1</td>
<td>7.6 ± 0.8</td>
<td>5.3 ± 2.1</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>(7489 ± 41)</td>
<td>201 ± 24</td>
<td>(660 ± 19)</td>
<td>(316 ± 18)</td>
<td>(51 ± 2)</td>
<td></td>
<td></td>
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<tr>
<td>5 min</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>83.1 ± 1.6</td>
<td>2.6 ± 0.6</td>
<td>8.2 ± 0.8</td>
<td>5.1 ± 1.8</td>
<td>0.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>(7511 ± 53)</td>
<td>214 ± 44</td>
<td>(738 ± 9)</td>
<td>(345 ± 17)</td>
<td>(38 ± 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 OSM</td>
<td>84.2 ± 1.5</td>
<td>2.3 ± 0.6</td>
<td>6.3 ± 0.7</td>
<td>4.6 ± 1.8</td>
<td>0.6 ± 0.2</td>
<td></td>
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<tr>
<td>(7360 ± 27)</td>
<td>259 ± 86</td>
<td>(549 ± 15)</td>
<td>(318 ± 14)</td>
<td>(38 ± 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6 OSM</td>
<td>85.3 ± 1.5</td>
<td>2.3 ± 0.5</td>
<td>5.5 ± 0.3</td>
<td>4.1 ± 0.9</td>
<td>0.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>(7557 ± 106)</td>
<td>179 ± 24</td>
<td>(505 ± 8)</td>
<td>(327 ± 52)</td>
<td>(50 ± 9)</td>
<td></td>
<td></td>
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</tbody>
</table>

* The numbers are the percentages of total [3H]inositol-labeled lipids and expressed as the means ± so of five or six values from three different experiments. The numbers in parentheses are the cpm of each inositol lipid and are the average of two values from one representative experiment.
IP₂ (data not shown). Thus, the decrease in [³H]PIP in stressed cells could have been partly caused by increased hydrolysis by PLC.

[³H]Inositol Phosphates in Intact Cells Did Not Change after Hyperosmotic Stress

The analysis of [³H]inositol phosphates in [³H]inositol-labeled plant cells is complicated by the large array of additional water-soluble metabolites (Loewus and Loewus, 1982; Rincón et al., 1989; Coté et al., 1990). It is very difficult to specifically assay [³H]inositol phosphates against this high background of [³H]-labeled compounds. In our experiments we have adapted an anion-exchange HPLC procedure that has been used successfully to resolve a number of inositol phosphates in extracts of animal cells (Balla et al., 1989; Menniti et al., 1990). The column was initially characterized with [³H]-labeled standards of 1-1-P, 1-4-P, IP₂, 1-1,3,4-P₃, IP₃, 1-1,3,4,5-tetrakisphosphate, and 1-hexaphosphate. Each sample also contained an internal standard of [⁴Cl]-1,3,4-P₃ to account for run-to-run variability.

The extract from control cells described in Figure 4 contained small [³H]-labeled peaks that coincided with the elution positions of 1-1-P and 1-4-P (combined total = 8089 dpm) plus IP₂ (1,464 dpm). IP₂ was not consistently detected. Two unidentified [³H] peaks that migrated between IP₂ and IP₃ and an unidentified [³H] peak between IP and IP₂ (all denoted with ?) were observed to increase in the cells treated at 0.6 osm. In these experiments the same fresh weight of cells was used for each treatment, and the total radioactivity recovered from each treatment was almost identical. There were no significant increases in [³H]PIP, [³H]IP₂, or [³H]IP₃ after subjecting cells to osmotic stress (0.4 or 0.6 osm) for either 5 min or 30 s (data not shown). Thus, we conclude that in the carrot cells stress-dependent increases in PLC-mediated hydrolysis of PIP are insufficient to alter the steady-state changes in levels of inositol phosphates to a substantial extent.

Hyperosmotic Stress Decreases PI Kinase Activity in Isolated Plasma Membranes

To determine whether or not there was a difference in PI kinase activity, plasma membranes were isolated from control and hyperosmotically treated cells. In vitro phosphorylation in the presence of [γ-³²P]ATP indicated that plasma membranes from hyperosmotically stressed cells formed 40% less [³²P]PIP than membranes from the control cells. This decrease in PIP formation was not due to limited substrate availability because a similar decrease in PIP formation was observed in the presence of exogenous PI (25 μg/each assay) (Table II).

When changes in PI kinase activity were monitored over time, slight but significant changes were observed consistently in plasma membranes from control cells (Fig. 5). The effect was transient and may be caused in part by a "touch"-induced response upon addition of the conditioned medium. More importantly at all times, the effect of osmotic stress was observed over and above that of the conditioned medium. After 2 min of hyperosmotic stress, there was a slight decrease in the amount of [³²P]PIP formed in the in vitro phosphoryl-
The elution time for ["4C"]1(1,3,4)P₃, an internal standard, is indicated and stressed cells. Cells were labeled with [3H]inositol (2.5 pCi
the PIP-PLC activity, the maximum decrease was found after
phorylation assay in which only endogenous substrate was
used, i.e. isolated plasma membranes alone, the major phos-
described in "Materials and Methods." The inositol phosphates
were identified by comparison to the elution times of standards.
phorylation product was phosphatidic acid. This is indicative
but remained at a low level (Fig. 5). In contrast to the PIP-PLC activity (Fig. 3), the activity of PI kinase did not recover by 10 min
remained at a low level (Fig. 5). In the in vitro phosphorylation assay in which only endogenous substrate was used, i.e. isolated plasma membranes alone, the major phosphorylation product was phosphatidic acid. This is indicative of the large amount of diacylglycerol in the plasma membranes. Lysophosphatidic acid, PIP, and an as yet unidentified phosphorylation product were also formed. PIP₂ was barely detectable, which is consistent with previous studies of plasma membranes from higher plants (Sandelius and Sommarin, 1986; Sommarin and Sandelius, 1988; Chen and Boss, 1990; Memon and Boss, 1990; Gross and Boss, 1992).

Pretreatment of Cells with Okadaic Acid Eliminates the Effect of Hyperosmotic Stress on PI Kinase and PIP-PLC Activity

The effect of hyperosmotic stress on PI kinase and PIP-PLC activity persisted in isolated plasma membranes. Because protein phosphorylation/dephosphorylation is a rapid and effective means of altering enzyme activity and because there was evidence for phosphatases decreasing PI kinase activity (Chen and Boss, 1990; Memon and Boss, 1990), we asked whether inhibiting dephosphorylation would affect the response of the membrane enzymes to hyperosmotic stress. For these experiments we used okadaic acid, a polyether fatty acid, that inhibits protein phosphatases 1 and 2A (Bialojan and Takai, 1988). Okadaic acid is hydrophobic and readily enters cells.

The effect of okadaic acid on the change in the PIP-PLC and PI kinase activity was studied both in vivo and in vitro. For the in vivo experiments cells were pretreated with okadaic acid (0.15 μM, final concentration) in DMSO or DMSO alone for 1 h. Okadaic acid pretreatment did not affect the plasmolysis (data not shown). As with the non-okadaic acid-pretreated cells, plasmolysis was complete within 30 s. In okadaic acid-pretreated cells, hyperosmotic stress no longer resulted in an increased PIP-PLC activity (Table III). It is interesting that okadaic acid pretreatment decreased the specific activity of PIP-PLC in both the control and hyperosmotic stressed cells to the same basal value (approximately 6 nmol min⁻¹ mg⁻¹ of protein).

Okadaic acid pretreatment also eliminated the difference

<table>
<thead>
<tr>
<th>Table II. The PI kinase activity of plasma membranes isolated from hyperosmotically treated and nontreated carrot suspension-culture cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>PI Kinase Activity</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>Using endogenous substrate</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Hyperosmotic</td>
</tr>
<tr>
<td>Using exogenous substrate</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Hyperosmotic</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
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<tr>
<td>a The data are the means ± SD of at least six values from three different experiments.</td>
</tr>
</tbody>
</table>

Figure 4. HPLC elution profile of inositol phosphates from control and stressed cells. Cells were labeled with ["3H"]inositol (2.5 μCi 0.1 
A) control; B, 0.4 osm; C, 0.6 osm). Total ["3H"]inositol phosphates were extracted and separated in HPLC as described in "Materials and Methods." The inositol phosphates were identified by comparison to the elution times of standards. The elution time for ["3C"]1(1,3,4)P₃, an internal standard, is indicated by the large arrow in each profile.
in PI kinase activity resulting from hyperosmotic stress. The PI kinase activity of plasma membrane from the okadaic acid-pretreated cells did not decrease when the cells were hyperosmotically stressed (Table IV). DMSO alone (the minus okadaic acid control) had no effect on the response to osmotic stress. It is interesting that adding okadaic acid to the plasma membranes isolated from hyperosmotically stressed cells decreased the specific activity of the PIP-PLC to approximately that of untreated membranes from control cells. Although the specific activity of the PIP-PLC decreased, the specific activity of PIP-PLC from hyperosmotically stressed cells remained higher than the okadaic acid-treated controls, i.e. adding okadaic acid to isolated plasma membranes did not overcome the effect of hyperosmotic stress.

**DISCUSSION**

We have shown that the metabolism of PIP changes as a result of hyperosmotic stress. After 5 min of stress there was a decrease in [3H]inositol-labeled PIP from whole cells. This was associated with a concomitant increase in PIP-PLC and a decrease in PI kinase-specific activity assayed in vitro. The observed changes in PIP metabolism did not correlate with the initial plasmolysis, which was complete within 30 s.

The fact that the effects of hyperosmotic stress on the

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>Assay Condition</th>
<th>PLC Activity (nmol min⁻¹ mg⁻¹)</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okadaic acid</td>
<td>-</td>
<td>Control</td>
<td>7.52 ± 0.82*</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>5.87 ± 0.08</td>
<td>78.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>Hyperosmotic</td>
<td>10.66 ± 0.48</td>
<td>141.7 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>5.63 ± 0.51</td>
<td>74.9 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td>12.83 ± 1.64</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>7.96 ± 0.86</td>
<td>62.0 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>Hyperosmotic</td>
<td></td>
<td>17.01 ± 1.16b</td>
<td>132.6 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>13.10 ± 0.92</td>
<td>102.1 ± 7.2</td>
</tr>
</tbody>
</table>

*The numbers are the averages ± SD of four values from two different experiments. The numbers are averages ± SD of three values from two experiments.
plasma membrane enzymes persisted in the isolated membranes indicated that the enzymes or some regulator of enzyme activity was altered. Although there is no evidence for covalent modification of PI kinase per se (Carpenter and Cantley, 1990), our data suggest that the changes in PIP metabolism observed as a result of hyperosmotic stress are mediated by an okadaic acid-sensitive phosphatase or by other okadaic acid-sensitive processes such as cytoskeleton reorganization (Fernandez et al., 1990) or intracellular transport (Lucocq et al., 1991).

Although PIP-PLC activity recovered to control values by 10 min, the PI kinase did not. These data suggest that, in these cells, PI kinase may be a more critical and sustaining factor for controlling steady-state PIP levels. Indeed, the PI kinase activity may reflect the physiological state of the cell. The decrease in the percentage of [3H]PIP and the tendency for the percentage of [3H]PPI to increase after 5 min of hyperosmotic stress would be consistent with activation of a PIP phosphatase (monoesterase) as well as a decrease in PI kinase activity; however, we could not detect PIP phosphatase activity in isolated plasma membranes assayed with endogenous [32P]PIP or exogenous [3H]inositol PIP as substrates (data not shown).

We started each experiment with the same fresh weight of cells. For each treatment, the inositol lipids and inositol phosphates were monitored as changes in cpm recovered as well as relative changes in the distribution of each lipid or inositol phosphate as a percentage of the total 3H recovered. [3H]PIP decreased both in cpm and as percentage of total 3H-lipid. Because the [3H]PIP was 10-fold higher than the [3H]-PPI and because the [3H]PPI was so low, it seems unlikely that an increase in the flux of PIP to PPI and IP2 makes a significant contribution to the decrease in [3H]PIP.

The profile of water-soluble 3H compounds indicates the complexity of inositol metabolism in plants, as others have shown (for review, see Coté et al., 1990; Loewus et al., 1990). We did not detect a net increase in [3H]PIP or [3H]PPI after osmotic stress. However, we could not exclude the possibility that the transient increase in PLC activity in vitro caused a limited increase in the rate of release in [3H]PPI or that osmotic stress enhanced the hydrolysis of IP2. Several laboratories have shown that the inositol phosphates are metabolized rapidly by plant homogenates and plant membranes and that the plant inositol phosphate phosphatases are not very sensitive to lithium (Loewus and Loewus, 1982; Drebak et al., 1988; Joseph et al., 1989; Memon et al., 1989b). Thus, newly released IP2 may have been quickly degraded to inositol.

In any event, within the limits of our measurements, inositol phospholipids do not appear to be involved in the primary signaling in response to hyperosmotic stress in carrot cells. It is possible that selective pools of PIP and IP2 are involved in the early signaling event; however, the magnitude of change certainly is not comparable to that of highly responsive animal cells such as the blowfly salivary gland (Berridge, 1983) or iris muscle (Akbart and Abdel-Latif, 1980). The change in PIP metabolism in the carrot cells appears to be secondary to the initial stimulus, perhaps prolonging or facilitating in this instance a change in cell state and ultimately reflecting a change in the metabolic state of the cell.

Einspahr et al. (1988) reported that increased [32P]PIP and [32P]PIP2 occurred as a result of short-term treatment of the halotolerant algae, D. salina, with NaCl. Based on these experiments we anticipated seeing an increase in [3H]inositol-labeled PIP and IP2 after hyperosmotic stress, but we found decreased recovery of [3H]PIP. The difference in the data might be due to differences in systems, halotolerant algae versus carrot cells, labeling positions, and, therefore, potential pools of labeled lipids, [32P]Pi versus [3H]inositol, and stress-inducing substances, NaCl versus sorbitol, respectively. Also, this discrepancy between algae and higher plants may be explained by the difference in regulation of PLC. For example, there is no evidence for effects of GTP or GTP·γ-S on the PLC-mediated breakdown of polyphosphoinositides in higher plants (Melin et al., 1987; McMurray and Irvine, 1988; Tate et al., 1989; Pical et al., 1992), but the activity of PLC

### Table IV. Effect of okadaic acid in vivo and in vitro on PI kinase activity

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>Assay Condition</th>
<th>PI Kinase Activity</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Okadaic acid</td>
<td>Control</td>
<td>-</td>
<td>261 ± 36.4*</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>216 ± 17.0</td>
<td>82.8 ± 9.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperosmotic</td>
<td>-</td>
<td>172 ± 23.8</td>
<td>65.8 ± 12.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>233 ± 23.2</td>
<td>89.3 ± 12.6</td>
<td></td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>Control</td>
<td>-</td>
<td>293 ± 21.7</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>250 ± 6.6</td>
<td>85.3 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Hyperosmotic</td>
<td>-</td>
<td>210 ± 11.7</td>
<td>71.7 ± 5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>166 ± 36.7</td>
<td>56.7 ± 12.5</td>
<td></td>
</tr>
</tbody>
</table>

* The numbers are the averages ± SD of four values from two different experiments.
the PLC-mediated breakdown of polyphosphoinositides in higher plants (Melin et al., 1987; McMurray and Irvine, 1988; Tate et al., 1989; Pical et al., 1992), but the activity of PLC was added to the lipid phosphorylation reaction mixture to give the final concentrations indicated. The PI kinase activity was assayed in the absence of exogenous substrate. Open circles, control; closed circles, hyperosmotic stress. The results are the means ± SD of four values from two different experiments.

Figure 6. The effect of okadaic acid in vitro on PI kinase activity in plasma membranes isolated from control and stressed cells. Cells were treated hyperosmotically (0.4 M) for 5 min as described in “Materials and Methods.” Okadaic acid (155 μM in 10% DMSO) was added to the lipid phosphorylation reaction mixture to give the final concentrations indicated. The PI kinase activity was assayed in the absence of exogenous substrate. Open circles, control; closed circles, hyperosmotic stress. The results are the means ± SD of four values from two different experiments.

the PLC-mediated breakdown of polyphosphoinositides in higher plants (Melin et al., 1987; McMurray and Irvine, 1988; Tate et al., 1989; Pical et al., 1992), but the activity of PLC from D. salina has been reported to be stimulated by the nonhydrolyzable GTP analog, GTP-γ-S (Einspahr et al., 1989).

Srivastava et al. (1989) studied the effects of mannitol on IP3 production in red beet slices. IP3 was measured as [3H]IP3 displaced from the IP3-binding protein. The amount of putative IP3 decreased 10 min after beet root slices were placed in 0.4 M mannitol and increased after 10 min in 0.2 M mannitol. If the red beet slices were plasmolyzed in 0.4 M mannitol, the decrease in IP3 would be consistent with the down regulation of the inositol lipid metabolism observed in the plasmolyzed carrot cells.

Salt stress resulted in reduced plasma membrane ATPase activity of tomato roots (Gronwald et al., 1990), suggesting that salt stress may impair the catalytic efficiency of the ATPase either by affecting the synthesis of positive or negative effectors or by affecting the lipid composition of the membrane. The carrot cell plasma membrane vanadate-sensitive ATPase activity also was reduced by hyperosmotic stress. The change in plasma membrane vanadate-sensitive ATPase activity previously has been shown to closely correlate with the PIP kinase activity in response to the cell-wall-degrading enzyme, Driselase (Chen and Boss, 1990). Results of the time-course study of ATPase and PI kinase activity in the osmotically stressed cells suggested that an initial decrease in ATPase activity may be unrelated to the level of PIP in plasma membranes; however, the later decrease in ATPase after 5 min may reflect the lower levels of PIP as the cells reach a new steady state. Negatively charged inositol lipids can directly affect the activity of P-type ATPases (Varsanyi et al., 1983; Schäffer et al., 1987; Memon et al., 1989a; Chen and Boss, 1991).

Within the context of signal transduction mechanisms the changes in inositol metabolism that we observed are slow and are probably down stream from the initial response. Slayman (1982) has shown that the plasma membrane of yeast cells depolarizes rapidly, within 30 s, in response to hyperosmotic stress, followed by a slower recovery period that lasts several minutes. Such a depolarization could indicate a change in ion flux or membrane structure. Stretch-activated ion channels have been shown to be present in tobacco cells grown in suspension culture and guard cells (Edwards and Pickard, 1987; Schroeder and Hedrich, 1989). These channels are activated by stretching the plasma membranes, and therefore, their open probability may be changed rapidly in response to mechanical stimuli or as a result of volume or turgor changes. Physiological responses within the cells could be mediated by a resultant increase in cytosolic calcium or by some other factors, such as a change in H+, K+, or Cl- ions. Calcium has been shown to activate PIP-PLC (Melin et al., 1987, 1992; Tate et al., 1989; Pical et al., 1992) and inhibit PI kinase activity (Kamada and Muto, 1992) in higher plants.

One consequence of decreased PIP is a potential loss of gelsolin or profilin binding and a resultant increase in actin severing and a decrease in actin nucleation sites (Janmey and Stossel, 1987; Janmey et al., 1987; Lind et al., 1987). In vitro studies indicate that profilin can bind to PIP and PIP2 and protect them from hydrolysis by PLC (Forscher, 1989; Goldschmidt-Clermont et al., 1990; Machesky et al., 1990). It is not yet clear, however, whether the increase in cytosolic calcium and the profilin release precede or follow PIP hydrolysis in vivo. The data suggest that profilin could link cell signaling at the membrane level to reorganization of the cytoskeleton. Based on these results we propose the following working model for carrot cells hyperosmotically stressed with sorbitol: Hyperosmotic stress induces membrane depolarization and plasmolysis within 30 s. An okadaic acid-sensitive phosphatase that is not associated with the plasma membrane is activated. After about 5 min the cells are in a recovery phase in which the cells begin to adjust to the new osmoticum. During the recovery phase, PIP-PLC is transiently activated while there is a sustained decrease in PI kinase activity. The result is a decrease in PIP. The net decrease in PIP would contribute to the decrease plasma membrane ATPase and affect cytoskeleton reorganization. As a result of these and
other intracellular events, the physiological state of cells changes, and cells reach a new steady state.

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LITERATURE CITED


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