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# *In Vitro* and *in Vivo* Phosphorylation of Polypeptides in Plasma Membrane and Tonoplast-Enriched Fractions from Barley Roots[1]

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## ABSTRACT

**Phosphorylation of polypeptides in membrane fractions from barley (*Hordeum vulgare* L. cv CM 72) roots was compared in *in vitro* and *in vivo* assays to assess the potential role of protein kinases in modification of membrane transport. Membrane fractions enriched in endoplasmic reticulum, tonoplast, and plasma membrane were isolated using sucrose gradients and the membrane polypeptides separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis. When the membrane fractions were incubated with γ-[$^{32}$P]ATP, phosphorylation occurred almost exclusively in the plasma membrane fraction. Phosphorylation of a band at 38 kilodaltons increased as the concentration of $Mg^{2+}$ was *decreased* from millimolar to micromolar levels. Phosphorylation of bands at 125, 86, 58, 46, and 28 kilodaltons required millimolar $Mg^{2+}$ concentrations and was greatly enhanced by $Ca^{2+}$. When roots of intact plants were labeled with [$^{32}$P]orthophosphate, polypeptides at approximately 135, 116, 90, 46 to 53, 32, 28, and 19 kilodaltons were labeled in the plasma membrane fraction and polypeptides at approximately 73, 66, and 48 kilodaltons were labeled in the tonoplast fraction. Treatment of the roots of intact plants with 150 millimolar NaCl resulted in increased phosphorylation of some polypeptides while treatment with 100 mM NaCl had no effect.**

Protein kinase activities are found in plasma membrane fractions from various plant tissues (1–4, 15, 21, 26–29). Although most studies of plant protein kinases have examined activities in purified plasma membrane fractions, a few studies indicate that kinase activity is also present in the tonoplast (2, 22, 24, 26, 27, 30, 31). Ion transport activities of both plasma membrane and tonoplast fractions from barley roots have been examined in detail (8–11, 13, 14). Because the assays for ion transport activity involve the addition of ATP to membrane fractions, it seemed likely that transport activities might be modulated by *in vitro* phosphorylation of the membrane proteins. For example, proton transport across the tonoplast and plasma membrane is differentially altered by including calcium in the proton transport assay (9); one explanation might be that addition of calcium stimulates a protein kinase in either membrane. Also, there are potential roles for *in vivo* modification of membrane proteins by protein kinases. For example, a sodium/proton antiporter was rapidly activated by exposing barley roots to NaCl (14).

To assess the potential role of protein kinases in regulation of ion transport in the isolated membranes, a detailed characterization of protein phosphorylation was carried out. For *in vitro* assays, ATP was added to isolated membrane fractions and the phosphorylation of endogenous membrane proteins was examined. The proteins phosphorylated *in vitro* were compared with those phosphorylated *in vivo*. When a kinase is isolated along with the membrane, the *in vitro* assays make it possible to manipulate the phosphorylation conditions. For example, evidence that a kinase is activated by $Ca^{2+}$ are easily obtained *in vitro*. The *in vivo* assay indicates which proteins are actually phosphorylated under physiological conditions and may detect the activity of soluble kinases that act on membrane proteins but are lost during isolation of the membranes. Isolation of membrane fractions before *in vitro* phosphorylation or after *in vivo* phosphorylation indicates the cellular location of the membrane-bound kinases and protein substrates and may facilitate their subsequent purification and identification.

In the work reported in this paper, barley root membrane proteins have been phosphorylated both *in vitro* and *in vivo* in order to examine which polypeptides are phosphorylated, their membrane location, and the conditions required for phosphorylation. The emphasis is on phosphorylation of endogenous substrates because of their potential role as membrane transport proteins.

## MATERIALS AND METHODS

### Plant Material

Seedlings of a salt-tolerant cv of barley (see ref. 16), *Hordeum vulgare* L. cv CM 72 (N. F. Davis Drier and Elevator, Inc. Firebaugh, CA), were grown above an aerated nutrient solution (12) at 22°C and 100% humidity for 6 to 7 d in darkness. The pH of the nutrient solution was maintained at 5.6 by daily addition of $Ca(OH)_2$.

### Membrane Preparation

Membrane fractions enriched in ER, tonoplast, and plasma membrane were isolated as described previously (11). The protein composition, lipid composition, activities of marker

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enzymes, ATPase activities, and ion transport activities of these fractions have been described in detail (6, 8–11, 13, 14, 19). The sample/22% (ER-enriched), 22/30% (tonoplast-enriched), and 34/40% (plasma membrane-enriched) interfaces were collected from a sucrose step gradient. The membranes were diluted 5- to 10-fold with buffer consisting of 250 mM sorbitol, 1 mM DTT, and 5 mM Mes adjusted to pH 7.0 with Tris, and centrifuged at 100,000*g* for 35 min. The pellets were suspended in a buffer consisting of 250 mM sorbitol, 1 mM DTT, 0.25 mM EGTA, and 25 mM Mes adjusted to pH 7.0 with Tris. Aliquots of the membranes were frozen at −70°C for up to 3 months. Protein was measured by the method of Bradford (5) using the Bio-Rad[2] dye-binding assay with bovine gamma globulin as the standard.

### *In Vitro* Phosphorylation for One-Dimensional PAGE

Phosphorylation was carried out at a protein concentration of 1.3 mg/mL in 50 μL of buffer containing 250 mM sorbitol, 1 mM DTT, 0.25 mM EGTA, and 25 mM Mes adjusted to pH 7.0 with Tris, plus $MgCl_2$ at the concentrations indicated in the text. For phosphorylation in the presence of $Ca^{2+}$, 0.35 mM $CaCl_2$ was added, resulting in a calculated free $Ca^{2+}$ concentration of 90 μM. The concentration of free $Ca^{2+}$ was calculated with a computer program, using the association constants for $CaEGTA^{2-}$, $MgEGTA^{2-}$, CaMes, etc., as described in Bush *et al.* (7). Phosphorylation was initiated by the addition of approximately 12.0 kBq of γ-[$^{32}$P]ATP (specific activity 111 TBq/mmol; New England Nuclear). Following incubation for 10 min at room temperature, the reaction was stopped by the addition of an equal volume of sample buffer containing 5% SDS, 40% glycerol, 8.8 M urea, 3.5% mercaptoethanol, 2 mM EDTA, and 90 mM Tris-HCl, pH 8.5. Samples were stored at −70°C until analyzed by PAGE. Protein loaded per lane was 20 to 23 μg.

For pulse-chase experiments, the reaction volume was increased to 150 μL. Unlabeled Tris-ATP (Sigma) was added to a final concentration of 2 mM and 25 μL aliquots taken at 0.5, 1, 2, and 5 min.

### pH Dependence of Phosphorylation

Plasma membrane was suspended in buffer consisting of 250 mM sorbitol, 1 mM DTT, 0.25 mM EGTA, and 5 mM Mes adjusted to pH 7.0 with Tris. The membranes were diluted approximately three-fold into buffer consisting of 250 mM sorbitol, 1 mM DTT, 0.25 mM EGTA, and 50 mM Mes adjusted with Tris to pH 5.3, 5.8, 6.2, 6.7, 7.2, or 7.7. The addition of the membranes did not cause major changes in the pH of the 50 mM Mes/Tris buffer. Because the assay includes excess $Ca^{2+}$ compared to EGTA, the change in pH from 5.3 to 7.7 resulted in a less than 1% change in the concentration of free $Ca^{2+}$ as calculated by the method of Bush *et al.* (7). Phosphorylation experiments were performed as described above except that the ATP used was from Amersham Corp.

[2] Mention of a specific product name by the U.S. Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

### *In Vivo* Phosphorylation

Twenty-one seedlings were placed in scintillation vials (three to four plants/vial) with 4 mL of nutrient medium and 1110 to 1850 kBq of $^{32}$Pi (Amersham) per vial and incubated overnight on a shaker. Roots were rinsed in three successive beakers of 25 mM K-phosphate (pH 6.8), excised, and tonoplast and plasma membrane fractions isolated as in Hurkman *et al.* (19). Membranes were washed in buffer consisting of 250 mM sorbitol, 1 mM DTT, 5 mM Mes adjusted to pH 7.0 with Tris, and 150 μM PMSF. The pellet was resuspended in 0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% (v/v) mercaptoethanol (pH 9.0). The proteins were extracted with phenol and precipitated as described in Hurkman and Tanaka (17), then dissolved in the same SDS buffer used for the *in vitro* samples. The total tonoplast or plasma membrane protein obtained from the 21 plants was loaded in one lane.

For some experiments, four to six seedlings were removed from the nutrient solution after labeling and immediately frozen in liquid nitrogen. The roots were broken off and microsomes were isolated but not separated into tonoplast and plasma membrane fractions. Potassium fluoride (50 mM) was added to the usual grinding buffer. The microsomes were extracted with phenol as described in Hurkman and Tanaka (17). In addition, protein was precipitated from an aliquot of postmicrosomal supernatant by adding a 5× volume of 0.1 M ammonium acetate in methanol. The supernatant protein was treated with 1 to 2 units of RNase A and RNase T1 for 10 min at 37°C before addition of the same SDS buffer used for the *in vitro* samples.

### One-Dimensional PAGE

SDS-PAGE was done according to the method of Laemmli (23). The polyacrylamide gels were 1.5 mm thick and consisted of a 12% separating gel and a 4% stacking gel. Samples were heated at approximately 80°C for 2 to 3 min before loading. Prestained mol wt standards (below) were run on each gel. The region of the gel ahead of the dye front, which contained the excess γ-[$^{32}$P]ATP, was discarded following electrophoresis. The gel was dried (Bio-Rad model 224 gel drier) and autoradiographs made using Kodak XAR-5 film with an intensifying screen (Du Pont Cronex Lightening Plus). Films were exposed at −70°C for 16 to 72 h.

Apparent molecular masses of phosphorylated polypeptides were calculated based on the mobilities of protein standards. A set of prestained proteins (Sigma) was used as standards: α-macroglobulin, 180 kD; β-galactosidase, 116 kD; fructose-6-phosphatase, 84 kD; pyruvate kinase, 58.0 kD; fumarase, 48.5 kD; lactic dehydrogenase, 36.5 kD; and triosephosphate isomerase, 26.6 kD. A set of standards (Sigma) that was not prestained was also run and then stained with Coomassie blue and compared with the prestained standards. This set of standards contained β-galactosidase, 116 kD; phosphorylase B, 97.4 kD; BSA, 66 kD; ovalbumin, 45 kD; lactate dehydrogenase, 36.2 kD; carbonic anhydrase, 29 kD; trypsin inhibitor, 20.1 kD; lactoglobulin, 18.4 kD; and lysozyme, 14.3 kD. The 58, 48.5, and 26.6 kDa prestained standards had lower mobilities than unstained standards of similar molecular mass.

Following autoradiography, some gels (see Fig. 1A) were rehydrated, by immersing the filter paper-backed dried gel in water, and then silver-stained as described in Hurkman and Tanaka (17).

### Two-Dimensional PAGE

For analysis by two-dimensional PAGE, proteins were phosphorylated *in vitro* as described for one-dimensional gels except that the sample volume was 200 μL and approximately 200 kBq Γ-[$^{32}$P]ATP was used. After 10 min, 200 μL of buffer containing 1.4 M sucrose, 1.0 M Tris, 60 mM HCl, 0.1 M EDTA, 0.2 M KCl, and 4% mercaptoethanol at pH 9.0 was added, followed by 400 μL of water-saturated phenol. Proteins were prepared for IEF[3] by the phenol-partitioning method exactly as described by Hurkman and Tanaka (17). Two-dimensional PAGE was done according to O'Farrell (25) with the modifications of Hurkman and Tanaka (17). The first-dimension IEF gels contained pH 3.5 to 10 and pH 5 to 7 (1:4, v/v) carrier ampholytes. Samples containing approximately 30,000 to 70,000 cpm were loaded on the acidic side of the IEF gels. The polypeptides in the IEF gels and a lane of prestained mol wt standards were separated on second-dimension SDS gels containing 10% polyacrylamide. The polypeptides were blotted to Immobilon P (Millipore) as described by Hurkman and Tanaka (18) and autoradiographs were made.

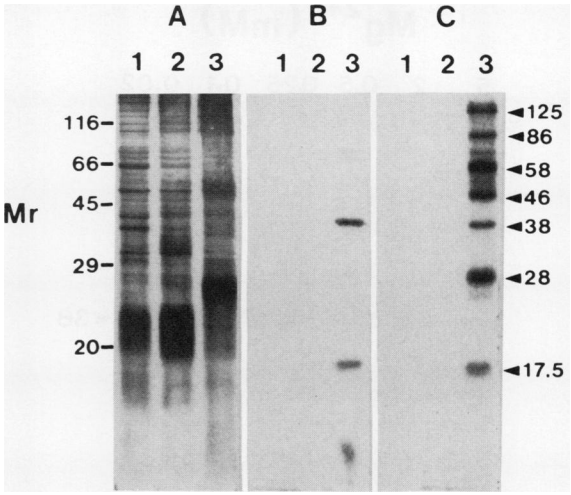


**Figure 1.** *In vitro* phosphorylation of polypeptides in ER, tonoplast, and plasma membrane fractions from barley roots in the presence of 5 mM $Mg^{2+}$. A, Silver-stained gel. B, Autoradiograph of polypeptides phosphorylated in the presence of 0.25 mM EGTA. C, Autoradiograph of polypeptides phosphorylated in the presence of EGTA plus 90 μM free $Ca^{2+}$. Lane 1, ER; lane 2, tonoplast; lane 3, plasma membrane. B and C were from the same SDS gel. A was a similar gel that was silver-stained to illustrate the number of polypeptides present in the ER and tonoplast fractions. Mol wt markers are shown for silver-stained gel only.

## RESULTS

### Comparison of *in Vitro* Protein Kinase Activities in Membrane Fractions from Barley Roots

Membrane fractions enriched in ER, tonoplast, or plasma membrane were labeled with γ-[$^{32}$P]ATP in the presence of 5 mM $Mg^{2+}$ and either the $Ca^{2+}$ chelator, EGTA (Fig. 1B), or EGTA plus 90 μM excess $Ca^{2+}$ (Fig. 1C). A silver-stained SDS gel (Fig. 1A) illustrates the many polypeptides present in each of the membrane fractions. However, relatively few polypeptides were phosphorylated and these were found almost exclusively in the plasma membrane fraction (lane 3, Fig. 1, B and C). The faint phosphorylated bands observed in the tonoplast fraction had the same mol wt as those in the plasma membrane fraction and were probably the consequence of slight contamination of the tonoplast fraction with plasma membrane. Little or no phosphorylation was observed in the ER fraction (lane 1, Fig. 1, B and C). The phosphorylation of polypeptides with molecular masses of 125, 86, 58, 46, and 28 kD in the plasma membrane fraction was greatly enhanced by added $Ca^{2+}$ (lane 3, Fig. 1C). In contrast, phosphorylation of polypeptides with molecular masses of 38 and 17.5 kD did not increase with added $Ca^{2+}$ (compare lane 3, Fig. 1, B and C).

### Effect of $Mg^{2+}$ on Protein Kinase Activity *in Vitro*

Preliminary results indicated that phosphorylation of some polypeptides changed greatly when the concentration of $Mg^{2+}$ was varied. Therefore, the effect of $Mg^{2+}$ concentration on phosphorylation of the membrane polypeptides was tested (Figs. 2, 3). Although only micromolar amounts of ATP were present, millimolar concentrations of $Mg^{2+}$ were required for the $Ca^{2+}$-stimulated phosphorylation of the plasma membrane polypeptides (Fig. 2). As the concentration of added $Mg^{2+}$ was reduced from 5 mM to 20 μM, the $Ca^{2+}$-stimulated phosphorylation of the polypeptides decreased. In contrast, phosphorylation of polypeptides at 38 and 17.5 kD, which did not require $Ca^{2+}$, greatly increased as the $Mg^{2+}$ concentration was reduced. This reduction in $Mg^{2+}$ concentration resulted in a less than 1% change in the free $Ca^{2+}$ concentration. To test whether phosphorylation of polypeptides in the ER and tonoplast might become visible at low $Mg^{2+}$ concentration, ER, tonoplast, and plasma membrane fractions were phosphorylated in the presence of 20 μM $Mg^{2+}$ either with (Fig. 3A) or without (Fig. 3B) $Ca^{2+}$. As in Figure 2, there was intense labeling of a 38 kD band in the plasma membrane fraction, with some labeling of a 17.5 kD band. However, there was still no phosphorylation of polypeptides unique to the ER or tonoplast. Faint labeling of a 38 kD band in the tonoplast and ER fractions was probably a consequence of slight contamination with plasma membrane. A 17.5 kD band was phosphorylated in the ER (compare Fig. 1B, lane 1 and Fig. 3B, lane 1), but the intensity of this band varied between experiments.

### Effect of $Ca^{2+}$ Concentration on Phosphorylation *in Vitro*

Phosphorylation of plasma membrane polypeptides was measured in the presence of 5 mM $Mg^{2+}$ (Fig. 4A) with 250

[3] Abbreviations: IEF, isoelectric focusing; pI, isoelectric point.

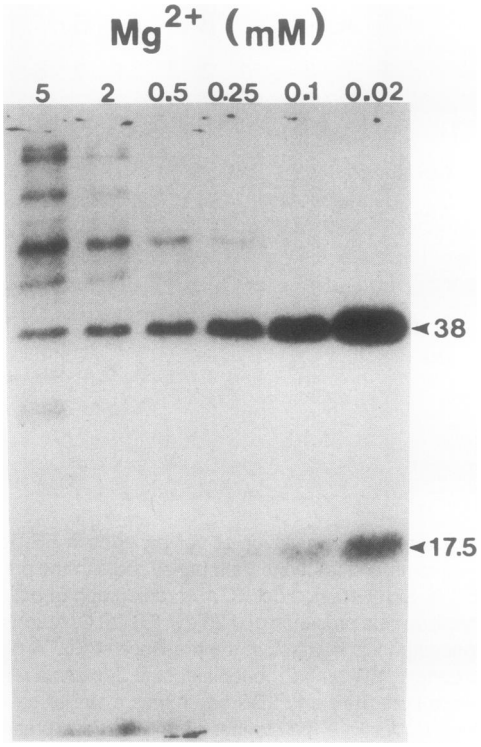


**Figure 2.** Effect of $Mg^{2+}$ concentration on *in vitro* phosphorylation of plasma membrane polypeptides of barley roots. Autoradiograph of plasma membrane polypeptides phosphorylated in the presence of EGTA plus 90 $\mu$M free $Ca^{2+}$ and the indicated concentrations of $Mg^{2+}$.

$\mu$M EGTA (lane 1), no additions (lane 2), or EGTA plus 90 $\mu$M excess $Ca^{2+}$ (lane 3). Without any added EGTA or $Ca^{2+}$ (lane 2), the $Ca^{2+}$ concentration in the reaction was estimated at 3 to 5 $\mu$M (10). This amount of $Ca^{2+}$ was already present in the water and reagents used to prepare the assay mix (10). At this lower $Ca^{2+}$ concentration, phosphorylated bands were observed in the same positions as in the sample phosphorylated in the presence of 90 $\mu$M excess $Ca^{2+}$. The intensity of most of the bands was similar at the two $Ca^{2+}$ concentrations, indicating that stimulation by $Ca^{2+}$ was saturated by 3 to 5 $\mu$M $Ca^{2+}$. However, phosphorylation of the 38 kD polypeptide was somewhat greater with 90 $\mu$M $Ca^{2+}$.

In the absence of any added $Mg^{2+}$ (Fig. 4B), increasing the $Ca^{2+}$ concentration from zero to 90 $\mu$M decreased the labeling of the 38 kD polypeptide (Fig. 4B). This was similar to the results in Figure 3, where the 38 kD polypeptide was most heavily phosphorylated at the lowest $Mg^{2+}$ and $Ca^{2+}$ concentration.

### Effect of pH on Phosphorylation of Plasma Membrane Polypeptides *in Vitro*

The effect of pH on phosphorylation of barley plasma membrane polypeptides was tested (Fig. 5). The plasma membrane fraction was incubated with $\gamma$-[$^{32}$P]ATP at various pH values in the presence of 90 $\mu$M excess $Ca^{2+}$ and 5 mM $Mg^{2+}$. Phosphorylation of the majority of the polypeptides, such as the 86 kD polypeptide, decreased as the pH was lowered from 7.7 to 5.3. Phosphorylation of the 38 kD and 17.5 kD polypeptides, however, increased as the pH was lowered.

### Treatment of Plasma Membrane with $\alpha$-[$^{32}$P]ATP

Labeling of polypeptides in the plasma membrane fraction was compared using ATP labeled at the $\alpha$- or $\gamma$-position to test whether the intense labeling of the 38 kD band that occurred with 20 $\mu$M $Mg^{2+}$ was the result of tightly bound ATP rather than phosphorylation (Fig. 6). When the membranes were labeled in the presence of only 20 $\mu$M $Mg^{2+}$, there was intense labeling of the 38 kD polypeptide with $\gamma$-[$^{32}$P]ATP (Fig. 6, lane 1) and very faint labeling with $\alpha$-[$^{32}$P]ATP (Fig.6, lane 2), indicating that the labeling was predominantly caused by phosphorylation with the $\gamma$-phosphate. Similarly, when the membranes were labeled in the presence of 5 mM $Mg^{2+}$, the labeling with $\alpha$-[$^{32}$P]ATP (Fig. 6, lane 4) was much fainter than that with $\gamma$-[$^{32}$P]ATP either in the presence (Fig. 6, lane 3) or absence (Fig. 6, lane 5) of $Ca^{2+}$. The faint labeling of the 38 kD polypeptide with $\alpha$-[$^{32}$P]ATP may be the result of a small amount of ATP binding to the polypeptide. If the labeling in lanes 2, 4, or 5 were caused by contaminating amounts of $\gamma$-[$^{32}$P]ATP in the $\alpha$-[$^{32}$P]ATP, or by synthesis of [-$^{32}$P]ATP from the [$\alpha^{32}$-P]ATP then other polypeptides in lane 4 should also have been labeled.

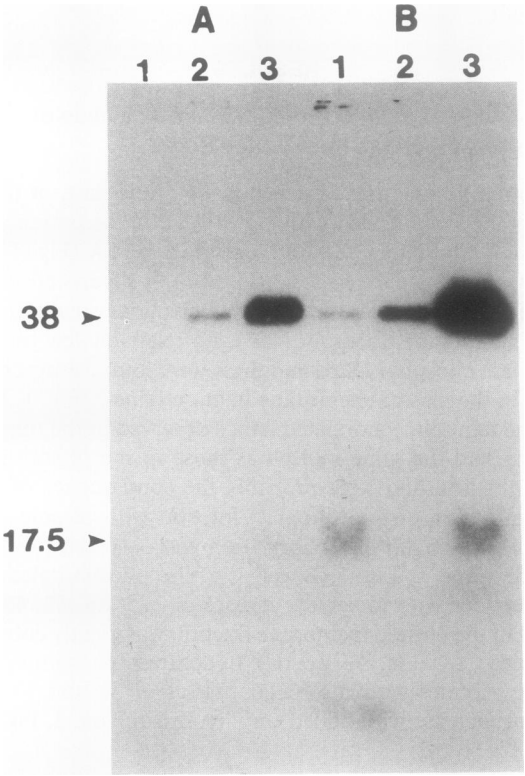


**Figure 3.** *In vitro* phosphorylation of polypeptides in ER, tonoplast, and plasma membrane fractions of barley roots in the presence of 20 $\mu$M $Mg^{2+}$. A, Autoradiograph of polypeptides phosphorylated in the presence of EGTA plus 90 $\mu$M free $Ca^{2+}$. B, Autoradiograph of polypeptides phosphorylated in the presence of 0.25 mM EGTA. Lane 1, ER; lane 2, tonoplast; lane 3, plasma membrane.

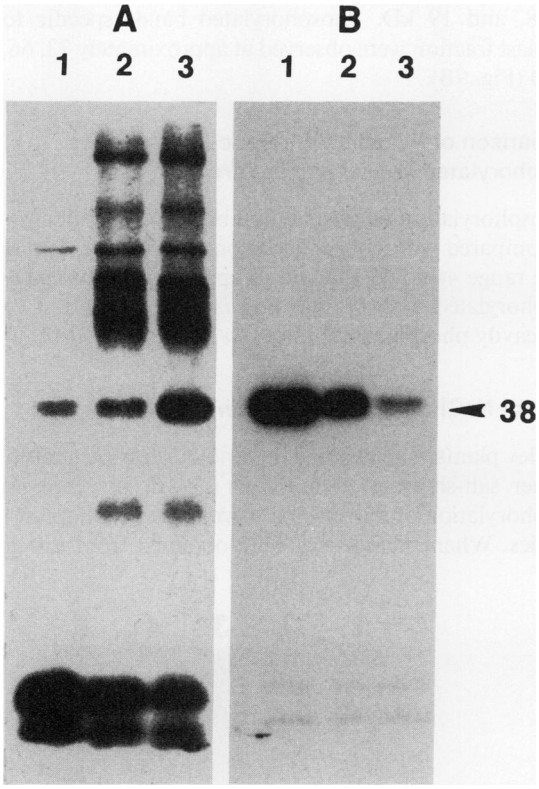


**Figure 4.** Effect of $Ca^{2+}$ concentration on *in vitro* phosphorylation of plasma membrane polypeptides of barley roots. A, Autoradiograph of plasma membrane polypeptides phosphorylated in the presence of 5 mM $MgCl_2$. B, Autoradiograph of plasma membrane polypeptides phosphorylated in the absence of $Mg^{2+}$. Lane 1, EGTA; lane 2, no additions, free $Ca^{2+}$ approximately 2 to 5 $\mu$M; lane 3, EGTA plus 90 $\mu$M free $Ca^{2+}$.

### Rate of Phosphate Turnover on the Polypeptides Phosphorylated *in Vitro*

Net phosphorylation in 10 min may represent a balance between protein kinase and phosphatase activities. To test for phosphatase activity in the plasma membrane fraction, a chase with unlabeled ATP was performed (Fig. 7). The plasma membrane fraction was labeled with $\gamma$-[$^{32}$P]ATP in the presence of 5 mM $Mg^{2+}$ and EGTA plus 90 $\mu$M excess $Ca^{2+}$. After 5 min of chase with the unlabeled ATP, the polypeptides whose labeling was stimulated by $Ca^{2+}$ showed little change. The chase with cold ATP eliminated the labeling of the 38 kD polypeptide, however. The chase eliminated labeling of 17.5 kD and 69 kD polypeptides in 30 s, the earliest time point sampled.

### Two-Dimensional PAGE of *in Vitro* Labeled Plasma Membrane

The relatively few bands in an SDS gel of the plasma membrane fraction from barley roots can be resolved into over 500 polypeptides in a two-dimensional gel (11, 19). To determine the number of unique polypeptides that were phosphorylated *in vitro* in the plasma membrane fraction, polypeptides were separated by two-dimensional PAGE following phosphorylation in the presence of $Ca^{2+}$ and low (20 $\mu$M) or high (5 mM) concentrations of $Mg^{2+}$ (Fig. 8). When the plasma membrane fraction was phosphorylated under low $Mg^{2+}$ conditions, only three polypeptides were detected by autoradiography (Fig. 8A). Each of these polypeptides had a molecular mass of 38 kD but had a different pI value. The polypeptide that incorporated the most label had a pI of approximately 6.4, with two minor spots at more acidic pIs. The 17.5 kD polypeptide, which also phosphorylated at low $Mg^{2+}$ concentrations, (Fig. 1B) was not detected. When the plasma membrane fraction was phosphorylated in the presence of a high concentration of $Mg^{2+}$, more than 20 phosphorylated polypeptides were detected by autoradiography (Fig. 8B). These polypeptides had molecular masses that ranged from 18 to 86 kD and pI values from 4.7 to 7.3. The correspondence between phosphorylated polypeptides and silver-stained polypeptides is indicated by arrows on the silver-stained gel (Fig. 8C). Comparison of the autoradiographs of the SDS and two-dimensional gels showed that some of the low mol wt phosphorylated polypeptides that were present in the two-dimensional gels did not correspond to phosphoprotein bands present in the SDS gels. In addition, the 17.5 kD and 125 kD bands that were present in SDS gels did not appear in the two-dimensional gels. This may be a consequence of differential solubilization of proteins by SDS compared with the

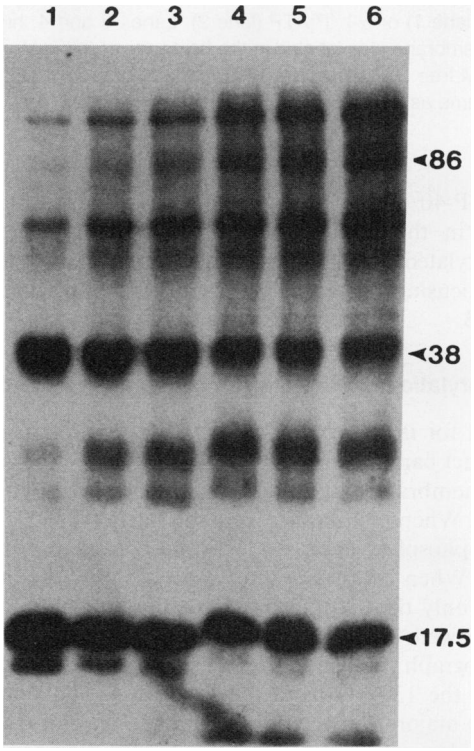


**Figure 5.** Effect of pH on *in vitro* phosphorylation of plasma membrane polypeptides of barley roots. Plasma membrane was phosphorylated in the presence of EGTA plus 90 $\mu$M $Ca^{2+}$ and 5 mM $Mg^{2+}$ at the following pH values: lane 1, 5.3; lane 2, 5.8; lane 3, 6.2; lane 4, 6.7; lane 5, 7.2; lane 6, 7.7.

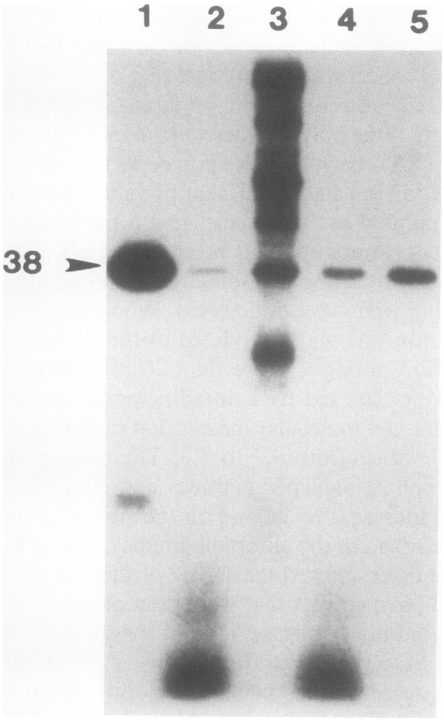


**Figure 6.** Comparison of *in vitro* phosphorylation of the plasma membrane fraction of barley roots by ATP labeled at the $\gamma$ or $\alpha$ position. Lanes 1 and 2, low $Mg^{2+}$. Plasma membrane was labeled in the presence of 20 $\mu$M $Mg^{2+}$ and EGTA plus 90 $\mu$M free $Ca^{2+}$ with $\gamma$-[$^{32}$P]ATP (lane 1) or $\alpha$-[$^{32}$P]ATP (lane 2). Lanes 3 and 4, high $Mg^{2+}$. Plasma membrane was labeled in the presence of 5 mM $Mg^{2+}$, EGTA plus 90 $\mu$M free $Ca^{2+}$ and $\gamma$-[$^{32}$P]ATP (lane 3) or $\alpha$-[$^{32}$P]ATP (lane 4). Lane 5, same as lane 3 but without $Ca^{2+}$.

Nonidet P-40 used in the first-dimension isoelectric focusing gels in the two-dimensional PAGE procedure. Also, phosphorylated polypeptides that did not enter the isoelectric focusing gel can be seen along the upper left side of Figure 8B.

### Phosphorylation of Membrane Polypeptides *in Vivo*

To test for *in vivo* phosphorylation of tonoplast polypeptides, intact barley plants were labeled with $^{32}$Pi for 20 h and plasma membrane and tonoplast fractions were isolated from the roots. When membranes were solubilized directly in SDS, a heavily phosphorylated band was observed at approximately 130 kD. When short exposures were used for the autoradiographs, only the 130 kD band was observed, and if longer exposures were used this band obscured a large portion of the autoradiograph (data not shown). Upon phenol extraction, however, the 130 kD band remained in the aqueous phase while the majority of the phosphorylated plasma membrane and tonoplast polypeptides partitioned into the phenol phase (Fig. 9). The 130 kD band was most enriched in the tonoplast fraction but was also observed in the plasma membrane fraction (Fig. 9A). Many phosphorylated bands were observed in the plasma membrane fraction, including bands at 135, 116, 90, 66, a diffuse band at approximately 49, and bands at 32, 28, and 19 kD. Phosphorylated bands specific to the tonoplast fraction were observed at approximately 73, 66, and 48 kD (Fig. 9B).

### Comparison of Plasma Membrane Polypeptides Phosphorylated *in Vivo* and *in Vitro*

Phosphorylation of plasma membrane polypeptides *in vivo* was compared with phosphorylation *in vitro* (Fig. 10). Bands in the range 46 to 53 kD and at approximately 87 kD were phosphorylated both *in vitro* and *in vivo*. A band at 28 kD was heavily phosphorylated both *in vivo* and *in vitro*.

### Effect of NaCl on Phosphorylation *in Vivo*

Barley plants were exposed to various salt treatments to test whether salt-shock or continuous growth in NaCl altered phosphorylation of any plasma membrane or tonoplast polypeptides. When membranes were obtained from salt-grown

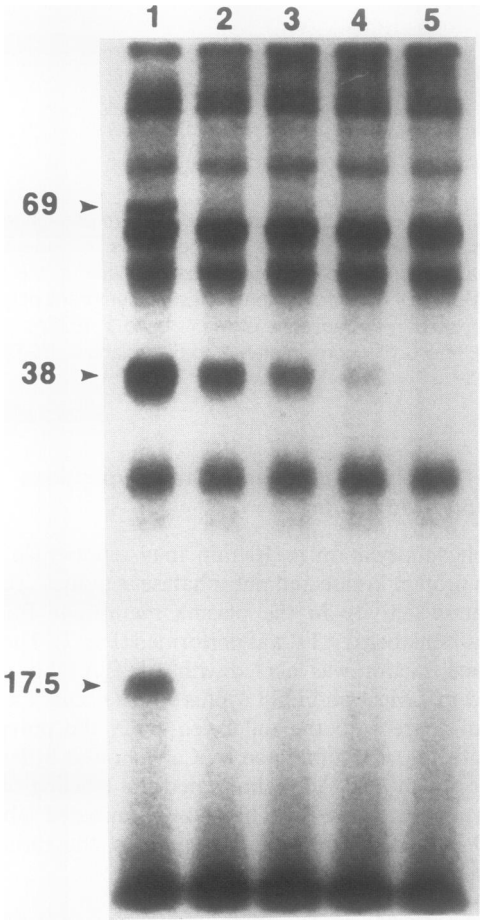


**Figure 7.** Pulse-chase analysis of phosphorylation of polypeptides in the plasma membrane fraction of barley roots. The plasma membrane fraction was phosphorylated in the presence of EGTA plus 90 $\mu$M free $Ca^{2+}$ and 5 mM $Mg^{2+}$. After 10 min, an aliquot was removed and mixed with sample buffer (lane 1). Unlabeled ATP (2 mM) was added and aliquots removed at the following times: lane 2, 0.5 min; lane 3, 1 min; lane 4, 2 min; lane 5, 5 min.

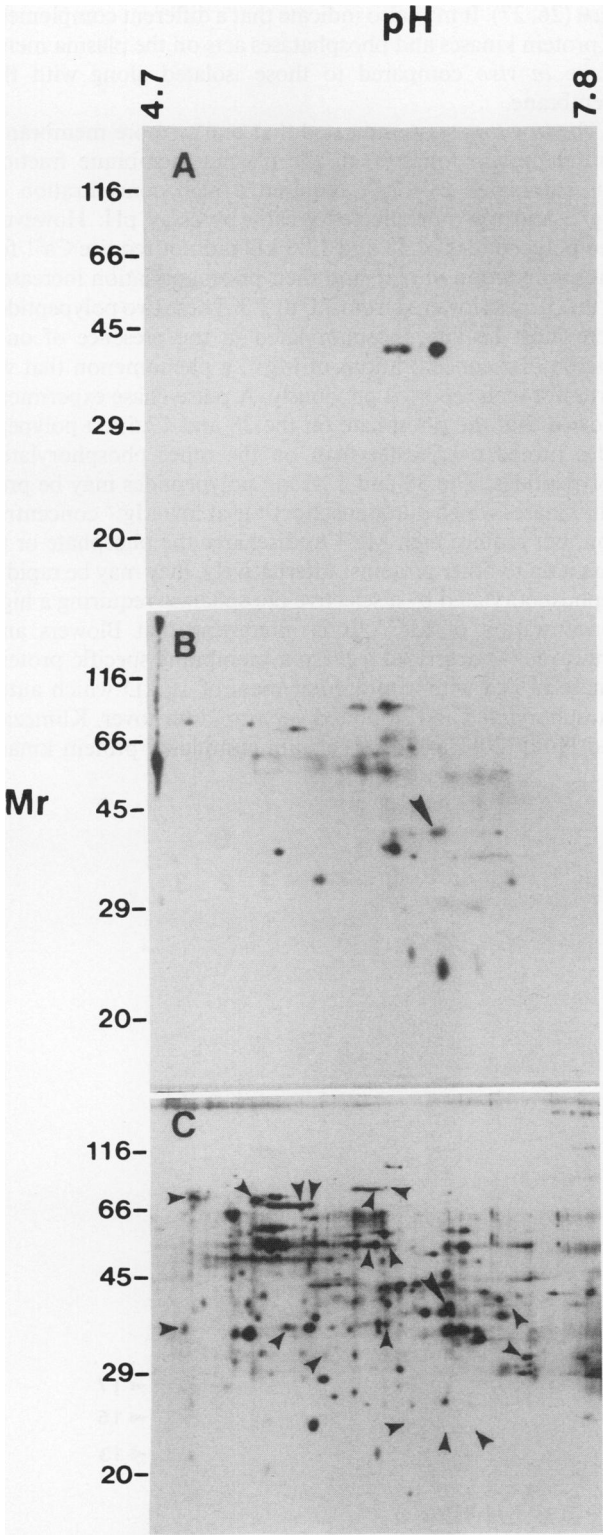


**Figure 8.** Two-dimensional gels of plasma membrane polypeptides of barley roots. A, Autoradiograph of blot showing polypeptides phosphorylated in the presence of EGTA plus 90 $\mu$M free $Ca^{2+}$ and 20 $\mu$M $Mg^{2+}$. B, Autoradiograph of blot showing polypeptides phosphorylated in the presence of EGTA plus 90 $\mu$M free $Ca^{2+}$ and 5 mM $Mg^{2+}$. Large arrow indicates the polypeptide phosphorylated in A. C, Silver-stained gel. Large arrow indicates the polypeptide phosphorylated in A; small arrows indicate the position of the phosphorylated polypeptides in B.

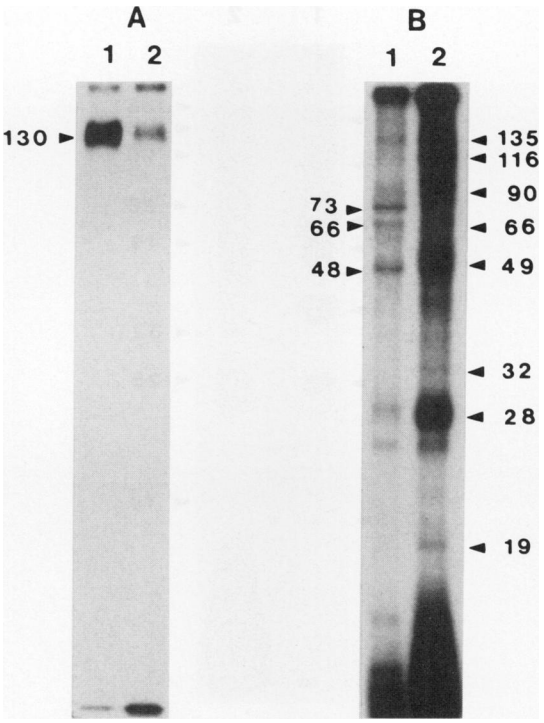


**Figure 9.** *In vivo* phosphorylation of polypeptides in the plasma membrane and tonoplast fractions from barley roots. The membranes were extracted by phenol partitioning and the phosphorylated products in the phenol phase and the aqueous phase were compared. A, Autoradiograph of phosphorylated polypeptides in the aqueous phase, separated on a 12% acrylamide gel. B, Autoradiograph of phosphorylated polypeptides in the phenol phase, separated on a 10% acrylamide gel. Lane 1, tonoplast; lane 2, plasma membrane.

or salt-shocked roots that had been treated with 100 mM NaCl, no difference in the phosphorylation of any polypeptides was detected by the *in vitro* assay. For the *in vivo* assay, roots were labeled with $^{32}Pi$ for 20 h, then subjected to a 10 min salt shock with 100 or 150 mM NaCl. Exposure to 100 mM NaCl did not alter phosphorylation of any of the polypeptides in the microsomal pellet or postmicrosomal supernatant (Fig. 11). However, a salt-shock with 150 mM NaCl resulted in a large increase in phosphorylation of polypeptides at approximately 17, 15, and 13 kD in the supernatant (Fig. 11). These polypeptides may also be present in the pellet, but the low mol wt range is partially obscured by other components, possibly lipids, in the autoradiograph of the microsomal fraction.

## DISCUSSION

A comparison of the *in vitro* phosphorylation of polypeptides in three well-defined membrane fractions from barley roots demonstrated that phosphorylation occurred in the

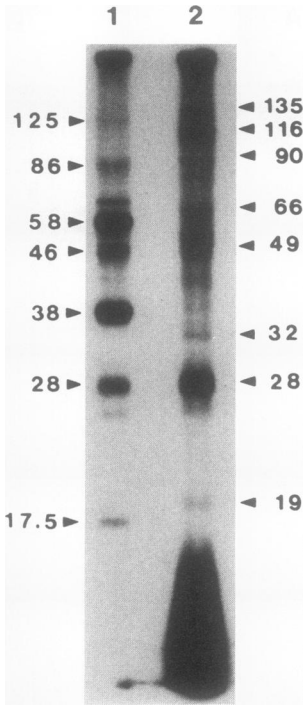


**Figure 10.** Comparison of *in vivo* and *in vitro* phosphorylation of plasma membrane polypeptides. Lane 1, autoradiograph of polypeptides phosphorylated *in vitro* in the presence of EGTA and 90 $\mu$M free $Ca^{2+}$. Lane 2, autoradiograph of polypeptides that were phosphorylated *in vivo*.

plasma membrane fraction and not in the endomembrane fractions. Such *in vitro* experiments only detect phosphorylation when both the kinase and substrate remain bound to the membranes during isolation. The *in vitro* assays indicated that one or more active protein kinases were isolated along with the plasma membrane-enriched fraction but not with the tonoplast-enriched fraction. Addition of ATP and $Mg^{2+}$ is used for assays of ion transport, such as assays of proton transport, sodium/proton exchange or calcium uptake into membrane vesicles (8–11, 13, 14). Clearly, proteins in the plasma membrane fraction from barley roots are being phosphorylated during the ion transport assays, whereas proteins in the tonoplast fraction are not. Analysis of the effects of $Mg^{2+}$, pH, calmodulin, and $Ca^{2+}$ on ion transport in the plasma membrane fraction from barley roots should take into account the possible role of protein phosphorylation. When plants were labeled *in vivo* with $^{32}Pi$, phosphorylated polypeptides were observed not only in the plasma membrane fraction but also in the tonoplast fraction. The prior state of phosphorylation of the membrane proteins might affect the results of ion transport assays with either membrane fraction.

The extent to which specific polypeptides in the plasma membrane-enriched fraction were phosphorylated was very different *in vivo* and *in vitro*. This suggests that the assay conditions used *in vitro* were significantly different from those experienced *in vivo*. For example, the lowest concentration of $Ca^{2+}$ used for the *in vitro* assay was 2 to 5 $\mu$M, whereas the normal concentration of calcium in the cytoplasm is less than 1 $\mu$M (26, 27). It may also indicate that a different complement of protein kinases and phosphatases acts on the plasma membrane *in vivo* compared to those isolated along with the membrane.

The *in vitro* assays indicated that one or more membrane-bound protein kinase(s) in the plasma membrane fraction was stimulated by $Ca^{2+}$, required a high concentration of $Mg^{2+}$, and was not affected greatly by assay pH. However, two polypeptides of 38 and 17.5 kD did not require $Ca^{2+}$ for phosphorylation *in vitro* and their phosphorylation increased as the pH was lowered from 7.0 to 5.3. These two polypeptides were most heavily phosphorylated in the presence of only micromolar concentrations of $Mg^{2+}$, a phenomenon that we have not seen reported previously. A pulse-chase experiment showed that the phosphate on the 38 and 17.5 kD polypeptides turned over faster than on the other phosphorylated polypeptides. The 38 and 17.5 kD polypeptides may be protein kinases which autophosphorylate at low $Mg^{2+}$ concentration, but require high $Mg^{2+}$ to discharge the phosphate or to pass it on to other proteins. Alternatively, they may be rapidly dephosphorylated by a selective phosphatase requiring a high concentration of $Mg^{2+}$. It is interesting that Blowers and Trewavas (4) described a plasma membrane specific protein kinase in pea with a molecular mass of 18 kD which autophosphorylated and exhibited very rapid turnover. Klimczak and Hind (20) purified a calcium stimulated protein kinase

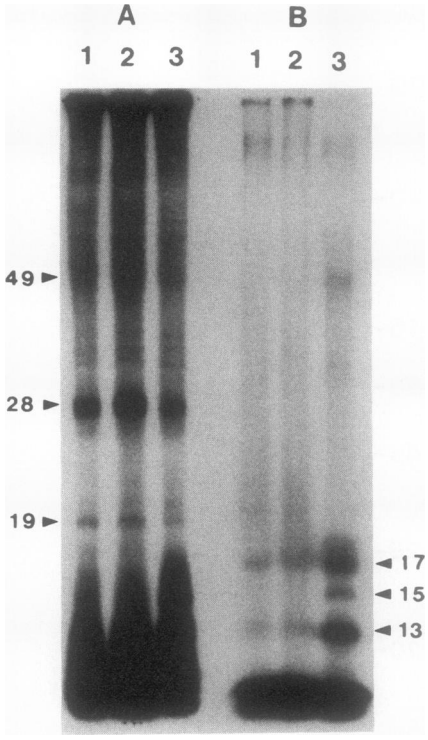


**Figure 11.** Effect of NaCl on *in vivo* phosphorylation of polypeptides in the microsomal pellet and postmicrosomal supernatant. Roots were frozen in liquid nitrogen, then homogenized. A, Microsomes; B, postmicrosomal supernatant. Lane 1, control; lane 2, roots were exposed to 100 mM NaCl for 10 min; lane 3, roots were exposed to 150 mM NaCl for 10 min.

from barley leaves with a mol wt of 37 kD on activity gels. Their data suggested that the same kinase was found in soluble and membrane fractions. It is possible that the 38 kD protein in the barley plasma membrane is related to or the same as the protein kinase described by Klimczak and Hind. However, the 38 kD protein is clearly associated with the root plasma membrane.

Analysis of polypeptides phosphorylated *in vitro* in the plasma membrane-enriched fraction revealed that only about 20 of the more than 500 polypeptides resolved in silver-stained two-dimensional gels were phosphorylated. The majority of the phosphorylated polypeptides corresponded to relatively minor spots in a silver-stained two-dimensional gel of the plasma membrane-enriched fraction. The most abundant plasma membrane polypeptides were not phosphorylated. In two-dimensional gels the 38 kD band was resolved into three spots which may represent the same polypeptide labeled with one, two, or three phosphates.

A $Na^+/H^+$ antiporter in tonoplast membranes is activated by exposing roots to 100 mM NaCl for 10 to 30 min (14). One mechanism for achieving a rapid, stable activation of a membrane transport protein is phosphorylation by a soluble or membrane-bound protein kinase. Therefore, we examined whether growth in salt or a salt-shock had any effect on phosphorylation of membrane proteins. Neither growth in salt or a salt-shock treatment with 100 mM NaCl had any effect on the phosphorylation of plasma membrane proteins assayed *in vitro* or *in vivo*. The only significant effect of salt-shock was a large enhancement of the phosphorylation of polypeptides at approximately 17, 15, and 13 kD when roots were exposed to 150 mM NaCl. This effect does not correlate with activation of the antiporter, since the antiporter was fully activated by exposure to 100 mM NaCl (14) but a salt shock with 100 mM NaCl was not sufficient to stimulate phosphorylation of the low mol wt polypeptides. Growth of barley roots is greatly depressed by exposure to 150, but not 100 mM NaCl, and the enhanced phosphorylation observed at 150 mM NaCl may be a response to cellular injury brought about by excess salt.

## SUMMARY

Phosphorylation of membrane proteins was compared *in vivo* and *in vitro* to assess the potential role of protein kinases in regulation of ion transport at the tonoplast and plasma membrane. Only plasma membrane proteins were phosphorylated *in vitro*. However, proteins in both the tonoplast and plasma membrane fractions were phosphorylated *in vivo*, indicating that protein kinases might be involved in modification of transport by both tonoplast and plasma membrane. Moderate concentrations of NaCl (100 mM) that activate a $Na^+/H^+$ antiporter in the tonoplast did not alter phosphorylation of plasma membrane or tonoplast proteins *in vivo* or *in vitro*. However, a salt shock with 150 mM NaCl stimulated the phosphorylation of low mol wt polypeptides in the microsomal fraction and in the postmicrosomal supernatant.

## LITERATURE CITED

1. **Blowers DP, Boss WF, Trewavas AJ** (1988) Rapid changes in plasma membrane protein phosphorylation during initiation of cell wall digestion. Plant Physiol **86:** 505–509
2. **Blowers DP, Hetherington A, Trewavas A** (1985) Isolation of plasma-membrane-bound calcium/calmodulin-regulated protein kinase from pea using Western blotting. Planta **166:** 208–215
3. **Blowers DP, Trewavas AJ** (1987) Autophosphorylation of plasma membrane bound calcium calmodulin dependent protein kinase from pea seedlings and modification of activity by autophosphorylation. Biochem Biophys Res Commun **143:** 691–696
4. **Blowers DP, Trewavas AJ** (1989) Rapid cycling of autophosphorylation of a $Ca^{2+}$-calmodulin regulated plasma membrane located protein kinase from pea. Plant Physiol **90:** 1279–1285
5. **Bradford M** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding. Anal Biochem **76:** 962–967
6. **Brown D, DuPont FM** (1989) Lipid composition of plasma membrane and endomembranes prepared from roots of barley (*Hordeum vulgare* L). Effects of salt. Plant Physiol **90:** 955–961
7. **Bush DS, Sticher L, Van Huystee R, Wagner D, Jones RL** (1990) The calcium requirement for stability and enzymatic activity of two isoforms of barley aleurone $\alpha$-amylase. J Biol Chem **265:** 19392–19398
8. **DuPont FM** (1987) Variable effects of nitrate on ATP-dependent proton transport by barley root membranes. Plant Physiol **84:** 526–534
9. **DuPont FM, Bush DS, Windle, JJ, Jones, RL** (1990) Calcium and proton transport in membrane vesicles from barley roots. Plant Physiol **94:**179–188
10. **DuPont FM, Hurkman WJ** (1985) Separation of the $Mg^{2+}$-ATPases from the $Ca^{2+}$-phosphatase activity of microsomal membranes prepared from barley roots. Plant Physiol **77:** 857–862
11. **DuPont FM, Tanaka CK, Hurkman WJ** (1988) Separation and immunological characterization of membrane fractions from barley roots. Plant Physiol **86:** 717–724
12. **Epstein E, Norlyn JD** (1977) Seawater-based crop production: a feasibility study. Science **197:** 249–251
13. **Garbarino J, DuPont FM** (1988) NaCl induces a $Na^+/H^+$ antiport in tonoplast vesicles from barley roots. Plant Physiol **86:** 231–236
14. **Garbarino J, DuPont FM** (1989) Rapid induction of $Na^+/H^+$ exchange activity in barley root tonoplast. Plant Physiol **89:** 1–4
15. **Hetherington A, Trewavas A** (1984) Activation of a pea membrane protein kinase by calcium ions. Planta **161:** 409–417
16. **Hurkman WJ, Fornari C, Tanaka CK** (1989) A comparison of the effect of salt on polypeptides and translatable mRNAs in roots of a salt-tolerant and a salt-sensitive cultivar of barley. Plant Physiol **90:** 1444–1456
17. **Hurkman WJ, Tanaka CK** (1986) Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol **81:** 802–806
18. **Hurkman WJ, Tanaka CK** (1987) The effects of salt on the pattern of protein synthesis in barley roots. Plant Physiol **83:** 517–524
19. **Hurkman WJ, Tanaka CK, DuPont FM** (1988) The effects of salt stress on polypeptides in membrane fractions from barley roots. Plant Physiol **88:** 1263–1273
20. **Klimczak LJ, Hind G** (1990) Biochemical similarities between

soluble and membrane-bound calcium-dependent protein kinases of barley. Plant Physiol **92**:919–923

21. **Klucis E, Polya GM** (1988) Localization, solubilization and characterization of plant membrane-associated calcium-dependent protein kinases. Plant Physiol **88:** 164–171
22. **Ladror US, Zielinski RE** (1988) Protein kinase activities in tonoplast and plasmalemma membranes from corn roots. Plant Physiol **89:** 151–158
23. **Laemmli UK** (1970) Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature **227:** 680–685
24. **Martiny-Baron G, Scherer GFE** (1989) Phospholipid-stimulated protein kinase in plants. J Biol Chem **264:** 18052–18059
25. **O'Farrell PH** (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021
26. **Poovaiah BW, Reddy ASN** (1987) Calcium messenger system in plants. Crit Rev Plant Sci **6:** 47–103
27. **Ranjeva R, Boudet AM** (1987) Phosphorylation of proteins in plants: regulatory effects and potential involvement in stimulus/response coupling. Annu Rev Plant Physiol **38:** 73–93
28. **Schaller GE, Sussman MR** (1988) Phosphorylation of the plasma-membrane $H^+$-ATPase of oat roots by a calcium-stimulated protein kinase. Planta **173:** 509–518
29. **Short TW, Briggs, WR** (1990) Characterization of a rapid, blue light-mediated change in detectable phosphorylation of a plasma membrane protein from etiolated pea (*Pisum sativum*) seedlings. Plant Physiol **92:** 179–185
30. **Surivastava A, Pines M, Jacoby B** (1989) Enhanced potassium uptake and phosphatidylinositol-phosphate turnover by hypertonic mannitol shock. Physiol Plant **77:** 320–325
31. **Teulieres C, Alibert G, Ranjeva R** (1985) Reversible phosphorylation of tonoplast proteins involves tonoplast-bound calcium-calmodulin dependent protein kinase(s) and protein phosphatase(s). Plant Cell Rep **4:** 199–201