Plant Physiol. (1992) 99, 1314–1320
0032-0889/92/99/1314/07/$01.00/0

Received for publication December 19, 1991
Accepted February 24, 1992

Syringomycin-Stimulated Phosphorylation of the Plasma Membrane H^+-ATPase from Red Beet Storage Tissue[1]

Yuka S. Suzuki, Yeelan Wang, and Jon Y. Takemoto*

Department of Biology and Program in Molecular Biology, Utah State University, Logan, Utah 84322–5305

ABSTRACT

The syringomycin-stimulated *in vitro* protein phosphorylation of the plasma membrane H^+-ATPase of red beet (*Beta vulgaris* L.) storage tissue was investigated. Peptides representing the H^+-ATPase N and C termini and nucleotide binding site (P-2, P-3, and P-1, respectively) were synthesized, and rabbit antisera against each were produced. In western immunoblots of purified plasma membranes, these antisera immunoreacted with the 100-kilodalton polypeptide of the H^+-ATPase and with other smaller polypeptides. The smaller polypeptides appeared to be degraded forms of the intact 100-kilodalton polypeptide. Immunoprecipitation experiments showed that plasma membranes treated with syringomycin had increased protein phosphorylation rates of the 100-kilodalton polypeptide. Optimal phosphorylation levels were achieved with 25 micromolar free Ca^{2+}. Phosphoserine and phosphothreonine were detected in the immunoprecipitates. Washed immunoprecipitates generated with anti-P-1 possessed protein phosphorylation activity. This immunoprecipitate activity was not stimulated by syringomycin, but it was inhibited when plasma membranes were treated with sodium deoxycholate before immunoprecipitation. The findings show that syringomycin stimulates the phosphorylation of the plasma membrane H^+-ATPase and that specific protein kinase(s) are probably associated with the enzyme.

SR[2] is a virulence factor produced by certain plant pathogenic strains of *Pseudomonas syringae* pv *syringae* (30). This 1226-D lipodepsipeptide (9, 28) affects the plasma membranes of plants and yeast (30). SR alters the charge potential and Ca^{2+} and K^+ fluxes across these plasma membranes (15, 25, 31, 34). The closure of stomata that it induces in whole leaves of *Vicia faba* (21) is consistent with its stimulation of cellular K^+ efflux (35). The reversal of SR-induced stomatal closure by fusicoccin shows that SR affects leaf guard cells without disrupting the plasma membrane (19).

SR stimulates the *in vitro* plasma membrane H^+-ATPase activities of the yeast *Rhodotorula pilimanae* and of red beet (*Beta vulgaris* L.) storage tissue (3, 34). It also stimulates the Ca^{2+}-dependent phosphorylation of several proteins of red beet storage tissue plasma membranes (2). Of special interest is the phosphorylation of one or more 100-kD polypeptides that correspond in size to the monomer of the P-type plasma membrane H^+-ATPase (2). Thus, SR may activate a protein kinase that phosphorylates and modulates the activity of the H^+-ATPase, or SR may inhibit protein dephosphorylation.

The identity of the 100-kD phosphopeptide with the H^+-ATPase has not been established, and it is not certain that SR stimulates the phosphorylation of the P-type H^+-ATPase. It is possible that SR could phosphorylate 100-kD polypeptides that have other functions. Dhugga and Ray (7) recently showed that major polypeptides of approximately 100 kD that are not P-type ATPases occur in plasma membranes of pea hypocotyls. A similar situation likely occurs with the red beet storage tissue plasma membranes. Thus, the 100-kD phosphopeptide must be identified as the H^+-ATPase before correlating its SR-stimulated activity with phosphorylation. Schaller and Sussman (27) reported evidence for the Ca^{2+}-dependent phosphorylation of the oat root plasma membrane H^+-ATPase. However, the phosphorylation was not induced by an effector and a relationship between phosphorylation and enzyme activity was not determined.

In this study, we used antisera against the H^+-ATPase to monitor its phosphorylation among the polypeptides of the red beet storage tissue plasma membrane. The antisera reacted with a 100-kD polypeptide, and SR-stimulated phosphorylation of this polypeptide was detected. Phosphothreonine and phosphoserine were found in an immunoreactive complex containing the phosphopeptide. These observations show that the phosphorylation rate of the red beet storage tissue plasma membrane H^+-ATPase increases with *in vitro* SR treatment.

MATERIALS AND METHODS

Red Beet Storage Roots and Isolation of Plasma Membranes

Red beet (*Beta vulgaris* L.) storage roots were purchased commercially. After removal of the leaves, the roots were stored in moist vermiculite at 4°C. Plasma membranes were isolated as described earlier (3). ATPase assays (3) were performed with all plasma membrane preparations, and the inhibition of ATPase activities by 100 μM sodium orthovanadate was measured. Only membrane preparations showing more than 80% inhibition were used.

[1] This research was supported by the National Science Foundation (grant DCB 8704077) and by the Utah Agricultural Experiment Station (project UTA00607). Y.S.S. was partly supported by a Utah State University Research Vice-President's Fellowship. This is UAES journal paper No. 4262.

[2] Abbreviations: SR, syringomycin; MAP, multiple antigenic peptide; CHAPS, 3-([3-cholamidopropyl)dimethylammonio]-1-propane sulfonate.

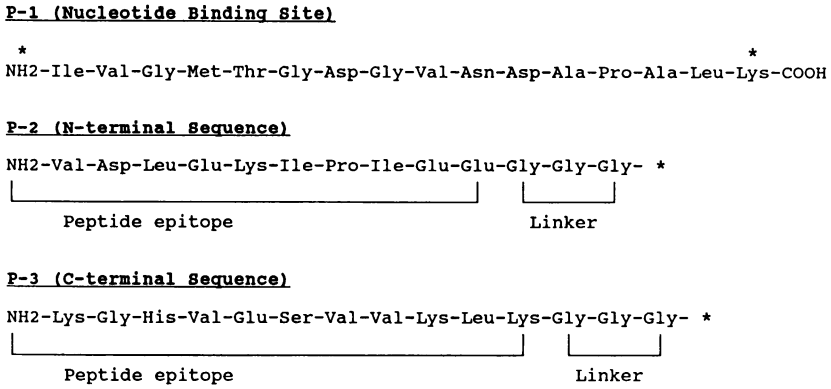

Figure 1. Amino acid sequences of the synthetic peptides. The nucleotide binding site, N-terminal, and C-terminal sequences (P-1, P-2, and P-3) were deduced from the gene sequence of the *A. thaliana* plasma membrane H^+-ATPase. Peptides were conjugated to the MAP system of Tam (35) and antisera against these peptide-MAP conjugates were obtained. Linkers between the MAP and P-2 and P-3 are indicated, and the cross-link positions between the lysyl group of the MAP and the peptides are shown by an asterisk (*).

Peptide Synthesis and Purified *Neurospora crassa* H^+-ATPase

Peptides P-1, P-2, and P-3 (Fig. 1) were synthesized at the Utah State University Biotechnology Center. P-1 was cross-linked to the lysyl group of the MAP system octavalent core of Tam (32) with glutaraldehyde. P-2 and P-3 were synthesized on the branching lysine core of the MAP system. Pure *Neurospora crassa* H^+-ATPase was provided by Dr. A. Bidwai (University of North Carolina).

Antisera

Control sera were obtained from rabbits before injection with antigens. Peptide or purified H^+-ATPase (1 mg) was mixed (1:1, v/v) with Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and injected into rabbits. Booster injections (1 mg) with Freund's incomplete adjuvant were given at 2-week intervals. Sera were obtained 2 weeks following each booster injection. Anti-oat H^+-ATPase serum directed against purified oat root H^+-ATPase was kindly given by Dr. M. Sussman (University of Wisconsin, Madison, WI).

Membrane Phosphorylation

Plasma membranes (25 μg protein) were added to 100 μL of phosphorylation reaction buffer (30 mM Tris-Mes [pH 6.5], 50 mM KCl, 15 mM $MgSO_4$, $CaCl_2$, and EGTA) with SR (20 μg SR/25 μg plasma membrane protein) or without SR. Typically, $CaCl_2$ and EGTA were added at concentrations to make 25 μM free Ca^{2+} according to the computerized calculations of Fabiato (8). To study the influence of Ca^{2+} concentration, the free Ca^{2+} levels were adjusted between 1 and 316 μM. The reaction was started by the addition of 7.5 nmol of ATP containing 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). Incubation was at 23°C for 30 min. For western immunoblotting experiments, each phosphorylation reaction was terminated by adding 2 volumes of quench solution containing 10% (w/v) TCA, 40 mM NaH_2PO_4, and 5 mM $Na_4P_2O_7$. Precipitated material was collected by centrifugation (13,000*g* for 5 min) and washed three times with the quench solution. Finally, the precipitates were washed with diethyl ether, to remove TCA, and dried. For immunoprecipitation experiments, each phosphorylation reaction was terminated by the addition of 100 μL of lysis buffer (150 mM NaCl, 1.0% [v/v] NP-40, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, 50 mM Tris-HCl [pH 8.0], 10 mM $Na_4P_2O_7$, and 80 mM NaH_2PO_4). For investigating the effects of various detergents, the phosphorylated plasma membranes were lysed with 100 μL of either 1% (w/v) CHAPS, 2% (w/v) octylglucoside, 2% (w/v) dodecylmaltoside, 1% (w/v) Zwittergent 3–10, 3–12, 3–14, or 3–16 or SDS at 1% or 0.5% (w/v), all in 10 mM Tris-HCl (pH 7.0), 0.1 M NaCl, and 20 mM EDTA instead of lysis buffer.

Western Immunoblotting

The phosphorylated plasma membrane polypeptides were treated with Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 10% [v/v] glycerol, 5% [v/v] 2-mercaptoethanol), loaded onto discontinuous 10% (w/v) polyacrylamide slab gels (1.5 mm thick) containing 0.1% (w/v) SDS, and electrophoresed according to Laemmli (16). After SDS-PAGE, proteins were electrotransferred to nitrocellulose membranes (Bio-Rad, 0.45 μm pore size). Immunoreactive polypeptides were detected using alkaline phosphatase-conjugated secondary antibodies and color development with nitroblue tetrazolium and bromochloroindoyl phosphate (10). The developed membrane was autoradiographed at −70°C with Kodak X-Omat AR film and intensifying screens.

Partial Purification of the H^+-ATPase

Plasma membranes (2 mg/mL) were solubilized with 20% (v/v) glycerol and 0.1% (w/v) Zwittergent 3–14 for 20 min at 4°C. The mixture was then centrifuged at 200,000*g* for 30 min at 4°C. The supernatant containing the ATPase activity was recovered.

Immunoprecipitation

Immunoprecipitation was performed using methods described by Harlow and Lane (10) with modification. All procedures were done at 4°C. The control serum (10 μL) was added to solubilized plasma membrane and incubated for 1 h with gentle agitation. Protein A agarose (50 μL, Immobilized rProtein A, RepliGen, Cambridge MA) was added and the reaction was incubated another 1 h. The mixture was then centrifuged at 13,000*g* for 1 min. The precleared supernatant was recovered and incubated with the antiserum (25 μL) and 0.1 mM PMSF overnight with gentle agitation. Protein

A agarose (50 μL) was added and incubated for 1 h. The immune complexes were centrifuged and washed three times with RIPA buffer (150 mM NaCl, 1.0% [v/v] NP-40, 0.1% [w/v] SDS, and 50 mM Tris-HCl [pH 8.0]). The pelleted materials were suspended in 50 μL of Laemmli sample buffer and incubated for 2 to 4 h. Phosphoproteins were separated by discontinuous 10% (w/v) SDS-PAGE and analyzed by autoradiography as described above.

Phosphoamino Acid Determination

Plasma membranes (50 μg of protein) were phosphorylated with [γ-^{32}P]ATP, lysed, immunoprecipitated, and treated with Laemmli sample buffer as described above. After centrifugation at 13,000*g* for 1 min, the supernatant fluid was recovered and BSA (100 μg) was added. Proteins were precipitated with 50 μL of 20% (w/v) TCA and the pellets were washed with diethyl ether to remove TCA. The dried proteins were suspended in 6 N HCl and hydrolyzed under vacuum at 110°C for 2 h. The hydrolysates were then dried under vacuum, dissolved in H_2O, and mixed with standard solutions of phosphoserine, phosphothreonine, and phosphotyrosine. Phosphoamino acids were separated by cellulose (0.1 mm) TLC at pH 3.5 (12). The developing solvent was *n*-butanol:isopropanol:formic acid:H_2O in a ratio of 3:1:1:1 (v/v/v/v). The plates were sprayed with 0.1% (v/v) triethylamine in acetone and then sprayed with ninhydrin (1 g/500 mL) to allow visualization of the amino acids. After drying, the plates were analyzed by autoradiography as described above for the immunoblots.

Phosphorylation of Immunoprecipitates

Unlabeled plasma membranes were disrupted with lysis buffer, with or without sodium deoxycholate, and immunoprecipitated with anti-P-1 as described above. The immunoprecipitates were then phosphorylated by incubation with [γ-^{32}P]ATP (5 μCi) in the phosphorylation reaction buffer (100 μL) at 23°C for 30 min. The labeled immunoprecipitates were washed three times with RIPA buffer, and the pelleted materials were analyzed by 10% (w/v) SDS-PAGE and autoradiography.

SR Purification

SR (E-form) was purified as described previously (3).

Protein Determination

The amounts of plasma membrane protein were determined by the method of Markwell *et al.* (17) using BSA as the standard.

Chemicals

[γ-^{32}P]ATP was purchased from Amersham Corp. (Arlington Heights, IL). The Zwittergent series of detergents, octylglucoside, and dodecylmaltoside were obtained from Calbiochem-Behring Co. (La Jolla, CA). CHAPS and SDS were from Boehringer Mannheim Co. (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Synthetic Peptides and Production of Specific Antisera

Rabbit antisera were produced against three synthetic peptides having primary structures deduced from the DNA sequences of the plasma membrane H$^+$-ATPase genes of *Arabidopsis thaliana* (11, 23). The peptides were: P-1, a 16-amino acid peptide representing the nucleotide-binding site and equivalent to peptide F of Schaller and Sussman (26); P-2, a 10-amino acid N-terminal peptide; and P-3, an 11-amino acid C-terminal peptide (Fig. 1). The P-1 sequence is highly conserved among P-type ATPases (11). The P-2 and P-3 sequences are unique to the plasma membrane H$^+$-ATPase and are conserved among the several isoforms of this enzyme (11). To assure high antibody titers, the peptides were cross-linked to or synthesized on the MAP system of Tam (32) before injection into rabbits.

Immunoreaction of the Peptide Antisera with the Red Beet Storage Tissue Plasma Membrane H$^+$-ATPase

SDS-PAGE western immunoblots of red beet storage tissue plasma membranes showed that all three peptide antisera reacted with a polypeptide of approximately 100 kD (Fig. 2A). Anti-P-2 and anti-P-3 reacted strongly with the 100-kD polypeptide; anti-P-1 reacted less strongly, and only weakly when the membranes were treated with SR and ATP. Other polypeptides reacted with the antisera. Polypeptides ranging in size between approximately 70 and 40 kD immunoreacted to varying degrees with the antisera. Polypeptides of about

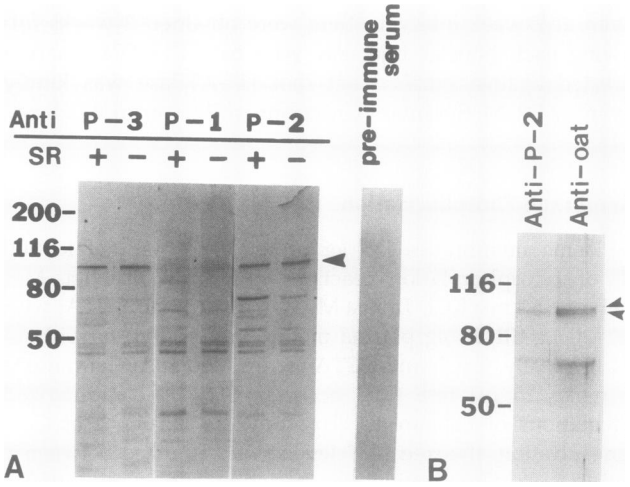

Figure 2. SDS-PAGE and western immunoblotting of the red beet storage tissue plasma membrane and H$^+$-ATPase. A, Membrane phosphorylation reactions were performed with or without SR and polypeptides were detected with anti-P-1, anti-P-2, or anti-P-3. The position of the immunoreactive intact 100-kD H$^+$-ATPase polypeptide is shown by an arrow. The lane on the far right shows immunoprecipitation with preimmune serum. B, Western immunoblots probed with anti-P-2 and antisera directed against the oat root plasma membrane H$^+$-ATPase show a similar pair of closely migrating 100-kD polypeptides (indicated by arrows). In B, the plasma membranes were not treated with SR.

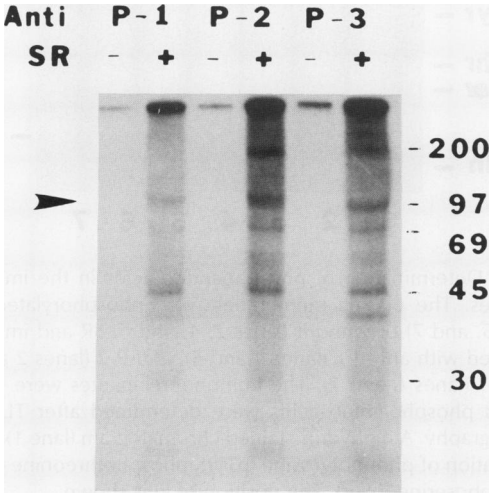

Figure 3. Autoradiogram of immunoprecipitated phosphopeptides. Plasma membranes were phosphorylated with or without SR, immunoprecipitated with anti-P-1, anti-P-2, or anti-P-3, washed, and subjected to SDS-PAGE. The position of the H+-ATPase 100-kD polypeptide is indicated by an arrow.

30 kD and smaller reacted weakly. Antisera directed against the oat root plasma membrane H+-ATPase gave the same western immunoblot band patterns as anti-P-2 (Fig. 2B). Both of these latter antisera revealed at least two closely migrating immunoreactive 100-kD bands. In contrast, antisera produced against the purified plasma membrane H+-ATPase of *N. crassa* gave a pattern resembling the one generated with anti-P-1 (data not shown). Incubation of the membranes with SR had no major effects on the polypeptide mobilities in SDS-PAGE (Fig. 2A). Anti-P-1 inhibited the activity of Zwittergent 3–14-solubilized and partially purified ATPase of the red beet plasma membrane by 35%.

Some of the immunoreactive polypeptides that were smaller than 100 kD appeared to be degradative products of the H+-ATPase. Purified membrane preparations having lower vanadate-sensitive ATPase activities showed more prominent immunoreactive bands of polypeptides with sizes between 70 and 40 kD. This observation is consistent with the well-known lability of the H+-ATPase.

When plasma membranes were incubated with [γ-^{32}P]ATP before SDS-PAGE and then western immunoblotted with anti-P-1, anti-P-2, or anti-P-3, a phosphopeptide corresponding to the immunoreactive 100-kD polypeptide was detected (data not shown). Other phosphopeptides were also observed. The phosphorylation of most of these polypeptides, including the 100-kD polypeptide, was stimulated by SR.

Immunoprecipitation of the H+-ATPase Phosphopeptides

The immunoblots described above suggested that the immunoreactive 100-kD H+-ATPase was phosphorylated and that SR stimulated the phosphorylation. However, with combined SDS-PAGE and western immunoblot analysis, the 100-kD H+-ATPase cannot be distinguished from similarly migrating, but functionally different, phosphopeptides. To overcome this problem, immunoprecipitation experiments were performed. Purified membranes were incubated with [γ-^{32}P]ATP with or without SR, solubilized, and the antigenic components immunoprecipitated in solution with anti-P-1, anti-P-2, or anti-P-3 serum. SDS-PAGE autoradiograms showed that the immunoprecipitates contained a major 100-kD phosphopeptide (Fig. 3). Other phosphopeptides having M_r values of 200,000, 70,000, 45,000, 30,000, and 20,000 were immunoprecipitated. All three anti-peptide sera gave the same phosphopeptide profile. Phosphorylation levels for all of the polypeptides were dramatically higher with SR treatment. Rabbit antisera against the plasma membrane H+-ATPase of oat roots or *N. crassa* gave similar immunoprecipitate phosphorylation patterns (data not shown). These observations indicate that the immunoreactive H+-ATPase 100-kD polypeptide was phosphorylated with SR treatment.

Effect of Ca^{2+} Concentration

The phosphorylation level of the immunoprecipitated polypeptides varied with the concentration of free Ca^{2+} (Fig. 4). Below 1 μM Ca^{2+}, phosphorylation was barely detectable. An optimal level was achieved at 25 μM Ca^{2+}.

Effect of Other Detergents on Immunoprecipitation

In the immunoprecipitation experiments described above, the plasma membranes were solubilized with a detergent mixture (lysis buffer) containing sodium deoxycholate, NP-40, and SDS. The immunoprecipitates contained phosphopeptides in addition to the intact H+-ATPase 100-kD phosphopeptide (Fig. 3). These other phosphopeptides were either immunoreactive derivatives of the H+-ATPase or non-H+-ATPase phosphopeptides that physically associated with the immunoprecipitating complex. In an attempt to dissociate and eliminate the non-H+-ATPase phosphopeptides, other solubilizing detergent solutions were substituted for lysis buffer. Octylglucoside (2% [w/v]), CHAPS (1% [w/v]), dodecylmaltoside (2% [w/v]), and Zwittergent 3–10, 3–12,

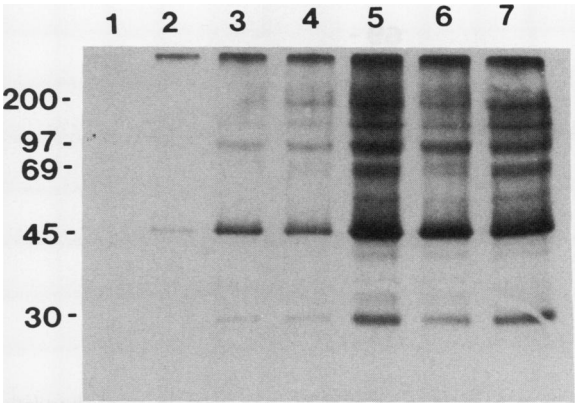

Figure 4. Autoradiogram showing the influence of free Ca^{2+} concentration on SR-stimulated H+-ATPase phosphorylation. The plasma membranes were phosphorylated in the presence of SR with 0 (lane 1), 1 (lane 2), 2.5 (lane 3), 7.9 (lane 4), 25 (lane 5), 100 (lane 6), and 316 μM (lane 7) free Ca^{2+} concentrations before lysis and immunoprecipitation with anti-P-1 serum.

3–14, and 3–16 (each 1% [w/v]) all gave phosphopeptide profiles like those typically observed with lysis buffer (data not shown). SDS solutions with concentrations of 0.5% (w/v) and 1% (w/v) inhibited the immunoprecipitation reaction. These observations indicated that if any non-H^+-ATPase phosphopeptides occurred in the immunoprecipitate, they were tightly associated with the immunoprecipitating complex.

Phosphorylation of the Immunoprecipitates

When anti-P-1 immunoprecipitates from lysis buffer-treated and unlabeled plasma membranes were incubated with [γ-^{32}P]ATP, the level of protein phosphorylation was relatively low. However, relatively prominent 30- and 20-kD phosphopeptides were observed (Fig. 5). When sodium deoxycholate was omitted from the lysis buffer, the immunoprecipitate phosphorylation rates were higher (Fig. 5). The phosphopeptide pattern resembled that obtained with phosphorylated membranes (Fig. 3). SR added to the immunoprecipitates during incubation with [γ-^{32}P]ATP did not alter the phosphopeptide patterns (data not shown). These observations show that a SR-insensitive, sodium deoxycholate-inhibited phosphorylation activity occurred in the immunoprecipitate.

Determination of the Phosphorylated Amino Acids

Immunoprecipitates from phosphorylated membranes were extracted and hydrolyzed with acid to determine which amino acids were phosphorylated. Autoradiograms of thin-layer cellulose chromatograms revealed that serine and, to a lesser extent, threonine were phosphorylated (Fig. 6). SR treatment of plasma membranes increased the phosphorylation level of both amino acids.

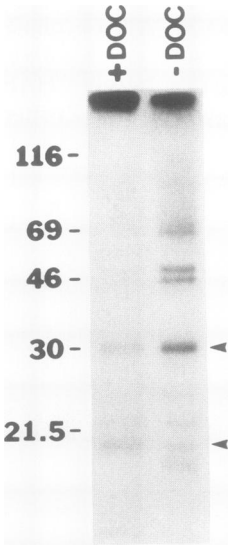

Figure 5. Autoradiograms showing the protein phosphorylation activities of the immunoprecipitates. Unlabeled plasma membranes were treated with lysis buffer with or without sodium deoxycholate and immunoprecipitated with anti-P-1 serum. The immunoprecipitates were phosphorylated with [γ-^{32}P]ATP and phosphopeptides were analyzed by SDS-PAGE and autoradiography. The positions of the 20 and 30-kD phosphopeptides are indicated by arrows.

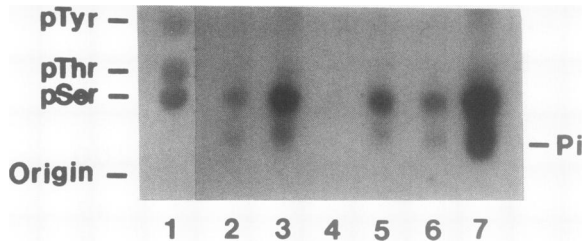

Figure 6. Determination of phosphoamino acids in the immunoprecipitates. The plasma membranes were phosphorylated with (lanes 3, 5, and 7) or without (lanes 2, 4, and 6) SR and immunoprecipitated with anti-P-1 (lanes 4 and 5), anti-P-2 (lanes 2 and 3), or anti-P-3 (lanes 6 and 7). The immunoprecipitates were hydrolyzed and phosphoamino acids were determined after TLC and autoradiography. A ninhydrin-stained chromatogram (lane 1) shows the separation of phophotyrosine (pTyr), phosphothreonine (pThr), and phosphoserine (pSer). The position of Pi is shown.

DISCUSSION

Our results show that the P-type H^+-ATPase of the red beet storage tissue plasma membrane is phosphorylated with SR treatment in a Ca^{2+}-dependent manner. It must be emphasized that this phenomenon occurs *in vitro* and that its *in vivo* occurrence and significance await further study. Nevertheless, an *in vivo* role for protein phosphorylation in SR's effects was recently shown with *Catharansus roseus* cells by Kauss and Jeblick (14). The SR-induced Ca^{2+} uptake in these cells was decreased by the addition of the protein phosphatase inhibitor okadaic acid. If and how plasma membrane H^+-ATPase phosphorylation plays a role in this effect is not known. The phosphorylation of the plasma membrane H^+-ATPase of oat roots (27) and yeast (33) have been reported previously. However, in both cases, phosphorylation was not modulated by effectors or correlated with enzyme activity.

Specific antibodies that immunoreact with the 100-kD polypeptide of the H^+-ATPase allowed us to monitor the phosphorylation of this enzyme. Several lines of evidence show that the immunoreactive 100-kD polypeptide is the H^+-ATPase: (a) Four different antisera immunoreacted with this polypeptide. These included one prepared against the purified oat root plasma membrane H^+-ATPase and two directed against the nonconserved N and C termini unique to the P-type H^+-ATPase (*i.e.* anti-P-2 and anti-P-3). (b) A decrease in the relative amount of the immunoreactive 100-kD polypeptide correlated with a decrease in the specific activity of the enzyme in plasma membrane preparations. (c) Anti-P-1 inhibited the activity of a vanadate-sensitive plasma membrane ATPase. (d) The 100-kD polypeptide was recovered in washed immunoprecipitates obtained with the specific H^+-ATPase antisera. Both the oat root H^+-ATPase antiserum and anti-P-2 detected two closely spaced polypeptide bands at the 100-kD region in SDS-PAGE, possibly reflecting the occurrence of isoforms of the H^+-ATPase (11) or proteolysis.

In addition to increasing the phosphorylation rate, SR also increases the *in vitro* activity of a plasma membrane-associated and vanadate-sensitive ATPase presumed to be the H^+-ATPase (2, 3). Initially, these two SR-induced phenomena suggested the possibility that increased phosphorylation of the enzyme leads to increased activity. However, other mechanisms for the SR-stimulated activity are possible. For example, the hydrolysis of phospholipids may be involved. Phospholipase-derived lipids such as phosphatidylinositol 5-monophosphate and phosphatidylinositol 4,5-bisphosphate stimulate the plasma membrane H^+-ATPase of sunflower hypocotyls and cultured carrot cells (18). Lysophosphatidylcholine stimulates ATP-dependent H^+-transport in oat root plasma membranes (20). Recent findings suggest that alteration of activity by these lipid derivatives may occur via interaction with an autoinhibitory C-terminal domain (21). It is possible that SR induces hydrolytic production of lipids that interact with the autoinhibitory domain to increase H^+-ATPase activity. Finally, SR may unmask latent H^+-ATPase activity by altering membrane permeability barriers and allowing access to the ATP substrate (22). Therefore, the mechanistic relationship between SR-stimulated H^+-ATPase phosphorylation and activity *in vitro* requires further study. In contrast, physiological observations with plants and yeasts suggest the possibility that *in vivo* SR may inhibit the plasma membrane H^+-ATPase. These observations include the SR-induced alkalinization of the yeast suspension medium (25) and leaf stomata closing that is associated with H^+-ATPase inhibition (19).

The serine and threonine-specific protein kinase activity observed in this study may be related to activities described by others. Recently, Putnam-Evans *et al.* (24) purified a soluble Ca^{2+}-dependent protein kinase from cultured soybean cells. It has been suggested that a membrane-bound form of Ca^{2+}-dependent protein kinase phosphorylates the plasma membrane H^+-ATPase (29). Because Ca^{2+}-dependent protein kinase phosphorylates both serine and threonine residues, a similar enzyme may be involved in the protein phosphorylations observed in the present work. Yanagita *et al.* (33) purified a protein kinase from plasma membranes of bakers' yeast that is stimulated by SR and other basic peptides. The phosphorylation sites were threonine and serine surrounded by glutamic acid residues (1). In our present work, however, SR did not appear to act directly on a protein kinase. Protein kinase activity in the immunoprecipitates was not influenced by SR. Also, the activities of protein kinases extracted from plasma membranes of red beet storage tissue were not influenced by SR (Y. Suzuki, J. Takemoto, unpublished data).

Immunoreactive polypeptides and phosphoproteins other than the intact 100-kD H^+-ATPase were observed. Some of the <100-kD phosphopeptides seen in immunoprecipitates were most likely truncated forms of the H^+-ATPase retaining the phosphorylation sites and specific antigenic domains. Plasma membrane preparations with lower ATPase specific activities had higher relative levels of <100-kD immunoreactive polypeptides. These smaller polypeptides resemble those observed in other plant plasma membrane H^+-ATPase preparations (13). The 200-kD phosphopeptide observed in the immunoprecipitates is possibly the dimeric form of the enzyme (6). Because of the presence of <100-kD phosphopeptides in the immunoprecipitates, it is not known if the H^+-ATPase is phosphorylated on serine or threonine or both.

Immunoprecipitates generated with anti-P-1 contained protein phosphorylation activity that was largely inhibited when membranes were lysed with sodium deoxycholate (Fig. 5). In the inhibited immunoprecipitates, 30- and 20-kD phosphopeptides were preferentially phosphorylated. Sodium deoxycholate is known to inhibit SR-stimulated ATPase and protein kinase activities (3). Therefore, these polypeptides are conceivably protein kinase components specifically associated with the H^+-ATPase. Polypeptides of similar size have been associated with plant plasma membrane protein kinases. A 29-kD polypeptide of sugar beet storage tissue plasma membrane that undergoes phosphorylation with ATP was described by Briskin and Thornley (5). Blowers and Trewavas (4) isolated an 18-kD Ca^{2+}-, calmodulin-dependent protein kinase from pea plasma membrane that is capable of autophosphorylation.

ACKNOWLEDGMENTS

We thank Drs. M.R. Sussman (University of Wisconsin) and A.P. Bidwai (University of North Carolina) for anti-oat root H^+-ATPase serum and purified *N. crassa* H^+-ATPase, respectively. We thank R.C. Bachmann and L. Takemoto for purifying SR, and Dr. T.A. Grover (Utah State University Biotechnology Center) for synthesizing peptides P-1, P-2, and P-3.

LITERATURE CITED

1. **Abdel-Ghany M, Raden D, Racker E, Katchalski-Katzir E** (1988) Phosphorylation of synthetic random polypeptides by protein kinase P and other protein-serine (threonine) kinases and stimulation or inhibition of kinase activities by microbial toxins. Proc Natl Acad Sci USA **85:** 1408–1411
2. **Bidwai AP, Takemoto JY** (1987) Bacterial phytotoxin, syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma membrane polypeptides. Proc Natl Acad Sci USA **84:** 6755–6759
3. **Bidwai AP, Zhang L, Bachmann RC, Takemoto JY** (1987) Mechanism of action of *Pseudomonas syringae* phytotoxin, syringomycin. Stimulation of red beet plasma membrane ATPase activity. Plant Physiol **83:** 39–43
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. **Briskin DP, Thornley WR** (1985) Plasma membrane ATPase of sugarbeet. Phytochemistry **24:** 2797–2802
6. **Briskin DP, Thornley WR, Roti-Roti JL** (1985) Target molecular size of the red beet plasma membrane ATPase. Plant Physiol **78:** 642–644
7. **Dhugga KS, Ray PM** (1991) Isoelectric focusing of plant plasma membrane proteins. Further evidence that a 55 kilodalton polypeptide is associated with β-1,3-glucan synthase activity from pea. Plant Physiol **95:** 1302–1305
8. **Fabiato A** (1988) Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. Methods Enzymol **157:** 378–417
9. **Fukuchi N, Isogai A, Yamashita S, Suyama K, Takemoto JY, Suzuki A** (1990) Structure of phytotoxin syringomycin produced by a sugar cane isolate of *Pseudomonas syringae* pv. *syringae*. Tetrahedron Lett **31:** 1589–1592
10. **Harlow E, Lane D** (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
11. **Harper JF, Manney L, Dewitt ND, Yoo MH, Sussman MR**

(1990) The *Arabidopsis thaliana* plasma membrane H^+-ATPase multigene. J Biol Chem **265:** 13601–13608

12. **Hunter T, Sefton BM** (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci USA **77:** 1311–1315
13. **Kasamo K** (1986) Purification and properties of the plasma membrane H^+-translocating adenosine triphosphatase of *Phaeseolus mungo* L. roots. Plant Physiol **80:** 818–824
14. **Kauss H, Jeblick W** (1991) Induced Ca^{2+} uptake and callose synthesis in suspension-cultured cells of *Catharanthus roseus* are decreased by the protein phosphatase inhibitor okadaic acid. Physiol Plant **81:** 309–312
15. **Kauss H, Waldmann T, Jeblick W, Takemoto JY** (1991) The phytotoxin syringomycin elicits Ca^{2+}-dependent callose synthesis in suspension-cultured cells of *Catharanthus roseus*. Physiol Plant **81:** 134–138
16. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
17. **Markwell MAK, Haas SM, Bieber LL, Tolbert NE** (1987) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem **87:** 206–210
18. **Memon AR, Chen Q, Boss WF** (1989) Inositol phospholipids activate plasma membrane ATPase in plants. Biochem Biophys Res Commun **162:** 1295–1301
19. **Mott KA, Takemoto JY** (1989) Syringomycin, a bacterial phytotoxin, closes stomata. Plant Physiol **90:** 1435–1439
20. **Palmgren MG, Sommarin M** (1989) Lysophosphatidylcholine stimulates ATP dependent proton accumulation in isolated oat root plasma membrane vesicles. Plant Physiol **90:** 1009–1014
21. **Palmgren MG, Sommarin M, Serrano R, Larsson C** (1991) Identification of an autoinhibitory domain in the C-terminal region of the plant plasma membrane H^+-ATPase. J Biol Chem **266:** 20470–20475
22. **Palmgren MG, Sommarin M, Ulvskov P, Larsson C** (1990) Effect of detergents on the H^+-ATPase activity on inside-out and right-side-out plant plasma membrane vesicles. Biochim Biophys Acta **1021:** 133–140
23. **Pardo JM, Serrano R** (1989) Structure of a plasma membrane H^+-ATPase gene from the plant *Arabidopsis thaliana*. J Biol Chem **264:** 8557–8562
24. **Putnam-Evans CL, Harmon AC, Cormier MJ** (1990) Purification and characterization of a novel calcium-dependent protein kinase from soybean. Biochemistry **29:** 2488–2495
25. **Reidl HH, Takemoto JY** (1987) Mechanism of action of bacterial phytotoxin, syringomycin. Simultaneous measurement of early responses in yeast and maize. Biochim Biophys Acta **898:** 59–69
26. **Schaller GE, Sussman MR** (1988) Isolation and sequence of tryptic peptides from the proton-pumping ATPase of the oat plasma membrane. Plant Physiol **86:** 512–516
27. **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
28. **Segre A, Bachmann RC, Ballio A, Bossa F, Grgurina I, Iacobellis NS, Pucci P, Simmaco M, Takemoto JY** (1989) The structure of syringomycins A1, E and G. FEBS Lett **255:** 27–31
29. **Sussman MR, Harper JF, Dewitt N, Schaller GE, Sheahan J** (1991) Plasma membrane cation pumps in *A. thaliana:* number of isoforms, expression and regulation of activity (abstract No. 1a). Plant Physiol **96:** S-1
30. **Takemoto JY** (1992) Bacterial phytotoxin syringomycin and its interaction with host membranes. *In* DPS Verma, ed, Molecular Signals in Plant-Microbe Communications. CRC Press, Boca Raton, FL, pp 247–260
31. **Takemoto JY, Giannini JL, Vassey T, Briskin DP** (1989) Syringomycin effects on plasma membrane Ca^{2+} transport. *In* A Graniti, RD Durbin, A Ballio, eds, Phytotoxins and Plant Pathogenesis. Springer-Verlag, Berlin-Heidelberg, pp 167–175
32. **Tam JP** (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc Natl Acad Sci USA **85:** 5409–5413
33. **Yanagita Y, Abdel-Ghany M, Raden D, Nelson N, Racker E** (1987) Polypeptide-dependent protein kinase from bakers' yeast. Proc Natl Acad Sci USA **84:** 925–929
34. **Zhang L, Takemoto JY** (1987) Effects of *Pseudomonas syringae* phytotoxin, syringomycin, on plasma membrane functions of *Rhodotorula pilimanae*. Phytopathology **77:** 297–303
35. **Zhang L, Takemoto JY** (1989) Syringomycin stimulation of potassium efflux by yeast cells. Biochim Biophys Acta **987:** 171–175