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Bacterial phytotoxin, syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma membrane polypeptides

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ABSTRACT **Syringomycin, a peptide toxin and a virulence factor produced by the bacterial phytopathogen *Pseudomonas syringae* pv. *syringae*, stimulated the phosphorylation of several plasma membrane polypeptides of red beet storage tissue. Among these was a 100-kDa polypeptide, which corresponds in size to the proton pump ATPase. The phosphorylations were insensitive to hydroxylamine, indicating that the polypeptide phosphorylated intermediates involved phosphate ester bonds characteristic of protein kinase-mediated phosphorylation. Phosphorylation of the 100-kDa polypeptide and of most of the other polypeptides was reduced or eliminated by extraction of the membranes with 0.1% (wt/vol) sodium deoxycholate, a treatment that also eliminated the ability of the toxin to stimulate ATPase activity. Phosphorylation of the 100-kDa polypeptide was highest with 10–20 μg of syringomycin; the same amounts gave the highest degree of ATPase activity stimulation. Phosphorylation of some of the polypeptides was eliminated or decreased by the Ca^{2+} chelator EGTA. Addition of excess Ca^{2+} restored the phosphorylation of most of these polypeptides. We conclude that syringomycin acts by stimulating an endogenous membrane protein kinase activity, which results in the phosphorylation of several membrane polypeptides. One of the phosphorylated polypeptides corresponds in size to the proton pump ATPase.**

Syringomycin is a peptide phytotoxin produced by many strains of *Pseudomonas syringae* pv. *syringae* (1). It is toxic to many plants and fungi and is implicated as a virulence factor in several major plant diseases. Among these are holcus spot disease of maize, bacterial canker of stone fruits, and brown leaf spot of beans (2).

Syringomycin appears to affect cell membranes, although the molecular details of its mode of action are unknown. Initial studies have shown that plasma membranes are a primary site of action (3, 4). The plasma membranes of the fungus *Geotrichum candidum* and of peach tree tissue were shown to be the predominant sites of toxin localization using autoradiographic and immunological methods (3). Recently, the effects of syringomycin on plasma membrane ion transport functions in yeasts were reported (5, 6). Syringomycin increased the charge potential (interior negative) and the pH gradient (interior alkaline) across the membrane and stimulated the plasma membrane ATPase. Comparisons to the effects on a respiratory deficient strain of *Saccharomyces cerevisae* indicated that mitochondria were not involved in the primary response to this toxin (6).

We have recently shown that syringomycin stimulates the vanadate-sensitive ATPase activity associated with the plasma membranes of red beet storage tissue (7). This enzyme is believed to operate as an electrogenic proton pump (8). The stimulation effect was lost after extraction of the membranes with 0.1% (wt/vol) sodium deoxycholate, and syringomycin had no effect on a partially purified ATPase preparation that was solubilized with Zwittergent 3-14. Since it was suggested that a protein kinase activity of the plasma membrane is reduced or eliminated by treating with 0.1% (wt/vol) sodium deoxycholate (9, 10), we speculated that syringomycin may modulate the ATPase activity through protein phosphorylation catalyzed by a kinase (7). Such a regulatory mechanism has been reported for the yeast plasma membrane ATPase (11) and the Ca^{2+}-ATPase of dog heart sarcolemma (12).

In plant cells, protein phosphorylation modulates many intracellular responses to Ca^{2+} and phospholipids (13–16). Recently, Tognoli and Colombo (17) reported the phosphorylation of cellular proteins induced by the fungal phytotoxin fusicoccin and demonstrated an increased phosphorylation of a 33-kDa polypeptide. Although fusicoccin is known to stimulate the plasma membrane ATPase (18, 19), a 100-kDa polypeptide corresponding in size to the ATPase (20–22) was evidently not phosphorylated.

In this report, we present evidence for the syringomycin-induced, protein kinase-mediated phosphorylation of several plasma membrane polypeptides of red beet storage tissue. Among these is a 100-kDa polypeptide, which we suggest to be the ATPase.

MATERIALS AND METHODS

Plant Material. Red beet (*Beta vulgaris* L.) storage roots were purchased commercially and after removal of the leaves were stored in moist vermiculite at 4°C.

Isolation of Plasma Membranes. Plasma membranes were isolated according to Briskin and Poole (20) with slight modifications. Beet roots were rinsed with distilled water and then peeled and cut into small sections. All subsequent steps were performed between 0°C and 4°C. Tissue (400 g) was homogenized in medium (400 ml) containing 250 mM sucrose, 3 mM EDTA, 0.5% polyvinylpyrrolidone (40 kDa), 70 mM Tris·HCl (pH 8.0), 30 mM mercaptoethanol, and 4 mM dithioerythritol in an Oster vegetable juice extractor. After filtration of the homogenate through four layers of cheesecloth, it was centrifuged at 13,000 × *g* for 15 min. The supernate was centrifuged at 30,000 × *g* for 30 min and the pellet was suspended in S buffer (250 mM sucrose/1 mM Tris-Mes, pH 7.2/1 mM dithioerythritol) to a final vol of 10 ml and homogenized with a glass Teflon Dounce homogenizer. An equal volume of S buffer containing 0.5 M KI was then added slowly, and the mixture was stirred gently on ice for 20 min. Then, the membranes were centrifuged at 80,000 × *g* for 30 min. The pellet was suspended in S buffer and layered onto a sucrose gradient consisting of 6 ml of 25% (wt/wt) sucrose layered over 5.5 ml of 34% (wt/wt) sucrose. The sucrose solutions were buffered with 1 mM Tris-Mes (pH 7.2) and 1 mM dithioerythritol. The gradients were centri-

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fuged at 115,000 × g for 2 hr. The plasma membranes were removed from the 25%/34% gradient interface, frozen under liquid nitrogen, and stored at −70°C.

Deoxycholate Extraction. Plasma membranes were adjusted to a protein content of 2 mg/ml using S buffer. The membranes were then treated with sodium deoxycholate as described (22). An equal volume of buffer containing 4 mM EDTA, 200 mM KCl, 50 mM Tris·HCl (pH 7.5), 1 mM dithioerythritol, and 0.2% (wt/vol) sodium deoxycholate (Sigma) was added slowly with mixing and then incubated for 20 min on ice. The membranes were harvested by centrifugation at 245,000 × g for 30 min, frozen under liquid nitrogen, and stored at −70°C.

Membrane Protein Phosphorylation. The reaction was carried out in a 1-ml vol containing 30 mM Tris-Mes (pH 6.5), 50 mM KCl, 40 μM $MgSO_4$, 2 mg of carrier bovine serum albumin (Sigma), and 300–400 μg of membrane protein. The reaction was initiated by the addition of 135 μmol of Tris ATP containing 25 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq) (ICN). Incubations were done at 7°C. Samples were removed at the appropriate times and mixed with 2 vol of quench solution containing 10% (wt/vol) trichloroacetic acid, 40 mM NaH_2PO_4, 5 mM $Na_2P_2O_7$, and 1 mM ATP. Uniform and rapid mixing was done by magnetic stir bars. The mixtures were centrifuged at 13,000 × g for 20 min. The supernatants were aspirated, and the pellets were resuspended in 500 μl of quench solution and recentrifuged again at 13,000 × g for 20 min. If needed, pelleted samples were then suspended in 250 mM hydroxylamine hydrochloride (pH 5.4) and incubated at room temperature for 20 min, after which 2 vol of quench solution was added and the mixtures were centrifuged at 13,000 × g for 20 min. All pellets were resuspended in 30 mM HCl and centrifuged again at 13,000 × g for 20 min. The HCl step selectively reduced the amount of bovine serum albumin in the samples without affecting the membrane protein content. The radioactivities in the resulting pellets were determined by liquid scintillation counting. Alternatively, portions of pelleted material (25 μg of protein) were each suspended in 50 μl of Laemmli sample buffer [62.5 mM Tris·HCl, pH 6.8/2% (wt/vol) $NaDodSO_4$/10% (vol/vol) glycerol/5% (vol/vol) 2-mercaptoethanol] and incubated at 50°C for 1–2 hr. Samples (25 μg of protein each) were loaded onto discontinuous 7% (wt/vol) polyacrylamide slab gels (1.5 mm thick) containing 0.1% $NaDodSO_4$ and electrophoresed according to Laemmli (23). After electrophoresis, the gels were stained in 0.04% (wt/vol) Coomassie brilliant blue R/10% (vol/vol) acetic acid/25% (vol/vol) isopropyl alcohol overnight and destained in 10% (vol/vol) acetic acid/10% (vol/vol) isopropyl alcohol. The destained gels were dried and autoradiographed at −70°C with Kodak XAR-5 film and intensifying screens.

ATPase Assay and Syringomycin Purification. ATPase assays were performed and syringomycin was purified as described (7).

Protein Assay. Protein was determined by the method of Markwell *et al.* (24).

RESULTS

Syringomycin-Induced Phosphorylation of Total Plasma Membrane Proteins. Using [^{32}P]ATP as substrate, the effect of syringomycin on the phosphorylation of red beet plasma membranes was investigated. Syringomycin (10 μg per 25 μg of protein) stimulated the total phosphoprotein levels ≈4-fold over controls (Fig. 1). The rate of phosphoprotein formation in the untreated controls reached a maximum between 5 and 7.5 min and then attained a constant level. With syringomycin, phosphorylation continued to increase even after 10 min.

Phosphorylation of Plasma Membrane Polypeptides. Gel electrophoretic and autoradiographic analyses of the phosphorylated plasma membranes revealed that syringomycin (10 μg per 25 μg of protein) stimulated the phosphorylation of several polypeptides (Fig. 2). The most dramatic phosphorylations were seen in gel bands B–H, corresponding to polypeptide sizes of approximately 140, 100, 70, 60, 50, 45, and 40 kDa, respectively. The 60-kDa polypeptide was quickly phosphorylated (within 15 s), and unlike other bands the level of phosphorylation appeared to decline after 2.5 min, implying a turnover of the phosphate. The 100-kDa polypeptide (C) corresponded in size to the catalytic subunit of the plasma membrane ATPase (20–22). A 200-kDa polypeptide (gel band A) was occasionally phosphorylated. It may possibly represent the dimeric form of the ATPase (21). Phosphorylation was typically performed at pH 6.5, but similar results were obtained at pH 7.0. Lower levels of phosphorylation occurred at pH 7.5.

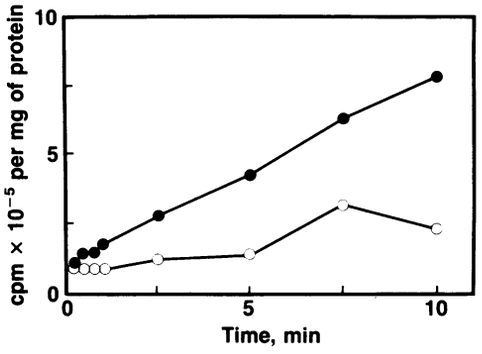

FIG. 1. Effect of syringomycin on the phosphorylation of red beet plasma membrane proteins. Plasma membranes (300 μg of protein) were incubated without (○) or with (●) 120 μg of syringomycin and the labeling of total protein by [^{32}P]ATP was determined.

Effect of Hydroxylamine Treatment on the Phosphoproteins. To distinguish between phosphoanhydride and phosphate ester bond formation, the phosphorylated samples were treated with hydroxylamine. Hydroxylamine selectively removes phosphates in anhydride bonds such as the aspartyl carboxyphosphate bond of the plant plasma membrane ATPase catalytic site (25, 26). The results show that hydroxylamine treatment did not alter the phosphate labeling pat-

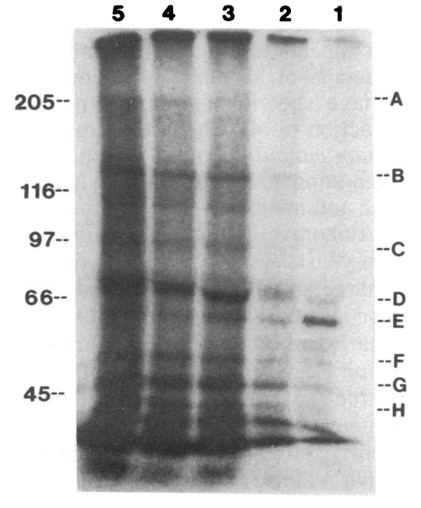

FIG. 2. Gel autoradiogram showing the time course of phosphorylation of plasma membrane polypeptides with syringomycin. The reactions were initiated in the presence of syringomycin (10 μg per 25 μg of protein) and stopped after 15 s, and 1, 2.5, 5, and 10 min (lanes 1, 2, 3, 4, and 5, respectively). The major phosphorylated polypeptides are indicated by letters on right. Positions of size standards are shown on the left (in kDa).

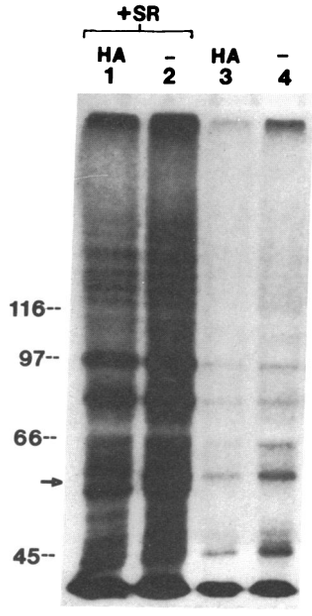

FIG. 3. Gel autoradiogram showing the effect of hydroxylamine (HA) treatment on plasma membrane phosphoproteins. The membranes were phosphorylated with (lanes 1 and 2) or without (lanes 3 and 4) syringomycin (SR) for 10 min. The samples were treated (lanes 1 and 3) or not treated (lanes 2 and 4) with hydroxylamine before gel electrophoresis. Arrow shows the position of ≈55-kDa polypeptide.

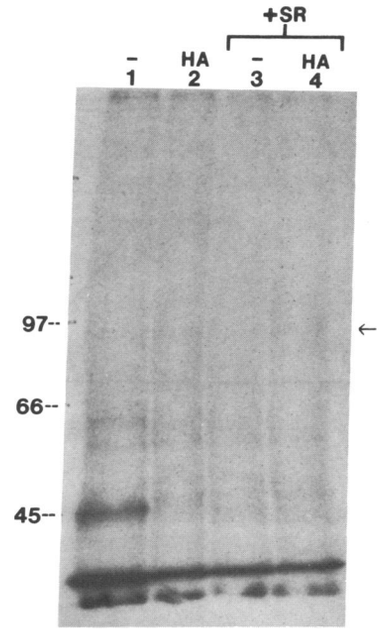

FIG. 4. Gel autoradiogram showing effect of sodium deoxycholate on the stimulation of the plasma membrane polypeptide phosphorylation by syringomycin (SR). Membranes were treated with 0.1% (wt/vol) sodium deoxycholate before assaying for ATPase activities (Table 1) and phosphorylation (10 min) in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of syringomycin. Samples were treated (lanes 2 and 4) or not treated (lanes 1 and 3) with hydroxylamine (HA) before gel electrophoresis. Arrow denotes the position of ≈100-kDa polypeptide.

terns of most of the polypeptides (Fig. 3). Radiolabeled phosphate was retained by most of the major phosphorylated polypeptides (i.e., the 140-, 100-, 70-, 60-, 50-, 45-, and 40-kDa polypeptides). The phosphate of an ≈55-kDa polypeptide was removed by hydroxylamine.

Effect of Sodium Deoxycholate on Syringomycin Stimulation of the Plasma Membrane ATPase Activity and Membrane Polypeptide Phosphorylation. As previously reported, sodium deoxycholate (0.1%, wt/vol) treatment eliminated the syringomycin-induced stimulation of the ATPase activity without inhibiting the enzyme (Table 1) (7). Phosphoprotein formation in the sodium deoxycholate-treated membranes was analyzed. Sodium deoxycholate treatment drastically reduced the phosphorylation of nearly all of the membrane polypeptides (Fig. 4). Significantly, the 100-kDa polypeptide suggested to be the ATPase was barely phosphorylated with syringomycin. One polypeptide, however, with an approximate mass of 45 kDa, was clearly phosphorylated in the sodium deoxycholate-treated membranes. The phosphate bond(s) formed with this polypeptide was sensitive to hydroxylamine, suggesting a phosphoanhydride bond, and its phosphorylation was inhibited by syringomycin.

Effect of Increasing Syringomycin Levels on Membrane

Table 1. Effect of sodium deoxycholate treatment on stimulation of red beet plasma membrane ATPase activity by syringomycin

Syringomycin, μg/ml	ATPase activity, μmol of P_i per hr per mg of protein	
	Untreated	Sodium deoxycholate treated
0	26.3 ± 0.11	76.1 ± 4.13
2.5	33.9 ± 9.43	68.1 ± 3.39
5.0	32.9 ± 0.11	66.1 ± 0.32
7.5	43.9 ± 5.83	65.4 ± 0.00
10.0	49.5 ± 2.05	64.0 ± 1.16

Assay mixture contained 15–25 μg of plasma membrane protein per ml. Values are means of triplicate determinations ± SD.

ATPase Activity and Polypeptide Phosphorylation. The stimulation of plasma membrane ATPase activity and hydroxylamine-resistant polypeptide phosphorylation increased in parallel with increasing amounts of syringomycin (Fig. 5). Phosphorylation of the 100-kDa polypeptide attained a maximum between 10 and 20 μg/ml and enzyme activity was highest between 15 and 20 μg/ml. At higher levels of toxin, both parameters declined. At 40 μg/ml and above, ATPase activities were inhibited below control values.

Effect of EGTA and Ca^{2+} on the Syringomycin-Stimulated Membrane Polypeptide Phosphorylation. Since previous reports (15, 16) demonstrated the regulation of plant protein kinases by Ca^{2+}, we investigated the role of Ca^{2+} in the enhanced protein phosphorylation observed with syringomycin. The Ca^{2+} chelator EGTA, at a concentration of 1 mM, eliminated the syringomycin-stimulated phosphorylation of several polypeptides (Fig. 6). This included the phosphorylation of the 140-, 60-, 50-, 45-, and 40-kDa polypeptides. A few polypeptides were partially affected (e.g., the 100- and 70-kDa polypeptides) or unaffected (e.g., a 55-kDa polypeptide) by EGTA treatment. Phosphorylation of most, but not all, of the polypeptides was restored by the addition of excess Ca^{2+}. Ca^{2+} (between 10 μM and 1 mM) alone had no effect on phosphorylation.

DISCUSSION

Our results show that syringomycin stimulated a plasma membrane-associated protein kinase activity responsible for the phosphorylation of several membrane polypeptides. Among those phosphorylated was a 100-kDa polypeptide, which corresponds in size to the proton pump ATPase (20). The phosphorylations were insensitive to hydroxylamine treatment, indicating that phosphoanhydride bond forma-

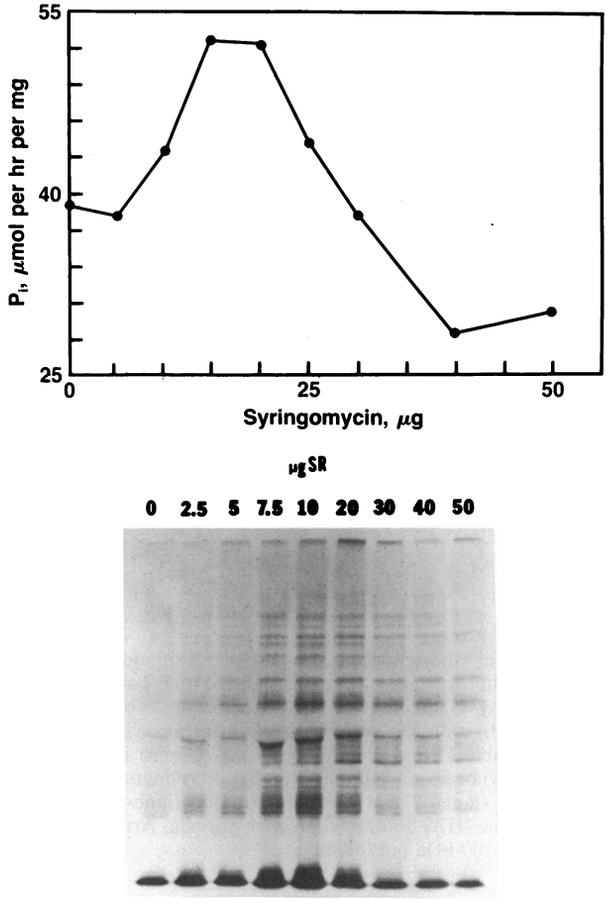

FIG. 5. Relationship between ATPase activities (*Upper*) and polypeptide phosphorylation (*Lower*) of red beet plasma membranes with increasing syringomycin (SR) levels. (*Lower*) Gel autoradiogram of phosphorylated samples after treatment with hydroxylamine and $NaDodSO_4$ gel electrophoresis. Arrow shows position of 100-kDa polypeptide.

tion, as with the carboxyl group of aspartic acid of the ATPase catalytic site, was not involved.

The data suggest (but do not prove) that phosphorylation causes increased ATPase activity. We previously showed that syringomycin stimulates the red beet plasma membrane ATPase activity and that this effect was lost upon extraction of the membranes with 0.1% (wt/vol) sodium deoxycholate (7). It was speculated earlier that sodium deoxycholate removed an endogenous membrane protein kinase activity (9, 10). In the present work, sodium deoxycholate treatment, which eliminated the stimulation of the ATPase activity, greatly reduced the phosphorylation of a 100-kDa polypeptide, which corresponds in size to the ATPase. Furthermore, a parallel increase and decrease between ATPase activity and phosphorylation of the 100-kDa polypeptide was observed with increasing syringomycin levels. Proof for modulation of the enzyme activity by phosphorylation will depend on demonstrating that the phosphorylated 100-kDa polypeptide is the ATPase.

A recent report on the fusicoccin-mediated phosphorylation of sycamore cell proteins did not show the phosphorylation of a 100-kDa polypeptide (17). This raises the possibility that fusicoccin and syringomycin may affect different protein kinase systems, and that the mechanisms by which they enhance the plant proton pump ATPase activity may differ.

Certain plant protein kinase activities are known to be regulated by Ca^{2+} (15), calmodulin (13), and Ca^{2+} plus phospholipids (27). Our results show that the Ca^{2+} chelator

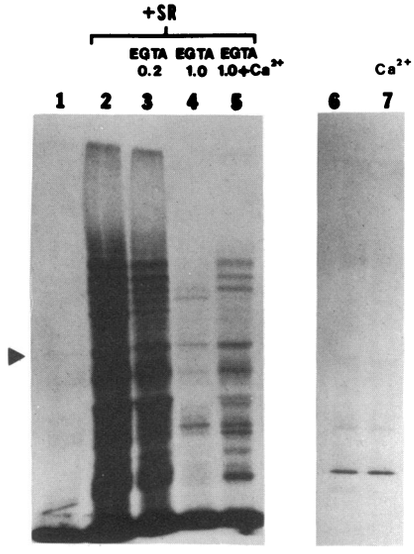

FIG. 6. Gel autoradiogram showing the effect of Ca^{2+} and EGTA on plasma membrane polypeptide phosphorylation stimulated by syringomycin (SR). Phosphorylations were conducted for 10 min without syringomycin (lanes 1 and 6), with 1 mM Ca^{2+} but no syringomycin (lane 7), with syringomycin (lane 2), with syringomycin + 0.2 mM EGTA (lane 3), with syringomycin + 1 mM EGTA (lane 4), and with syringomycin + 1 mM EGTA + 2 mM Ca^{2+} (lane 5).

EGTA removes the ability of syringomycin to induce the phosphorylation of certain plasma membrane polypeptides (Fig. 5). Addition of excess Ca^{2+} to the reaction mixture containing EGTA restored the capacity of the toxin to induce phosphorylation. However, the phosphorylation of a few polypeptides was less sensitive to EGTA, suggesting that Ca^{2+} involvement is complex and that perhaps multiple protein kinases, which are sensitive to syringomycin, occur in red beet plasma membranes. Multiple protein kinase activities in plants have been observed (13–16, 27).

We conclude that syringomycin exerts its effects by activating a membrane-bound protein kinase activity, which results in the phosphorylation of several plasma membrane polypeptides. We speculate that among these is the proton pump ATPase, which turns over at higher rates upon phosphorylation.

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