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Mechanism of Action of *Pseudomonas syringae* Phytotoxin, Syringomycin[1]

STIMULATION OF RED BEET PLASMA MEMBRANE ATPase ACTIVITY

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ABSTRACT

Syringomycin, a peptide toxin produced by the phytopathogen *Pseudomonas syringae* pv *syringae* preferentially stimulated (2-fold) the vanadate-sensitive ATPase activity associated with the plasma membrane of red beet storage tissue. The toxin had a very slight effect on the tonoplast ATPase and had no detectable effect on the mitochondrial ATPase. Optimal stimulation was achieved with 10 to 50 micrograms of syringomycin per 25 micrograms of membrane protein. Treatment of membranes with 0.1% (weight/volume) deoxycholate eliminated the activation effect, and enzyme solubilized with Zwittergent 3-14 was not affected by syringomycin. ATPase activity was activated to the same extent at KCl concentrations ranging from 0 to 50 millimolar. Valinomycin, nigericin, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, and gramicidin did not increase the plasma membrane ATPase activity. However, these ionophores did not hinder the ability of syringomycin to stimulate the activity. We suggest that syringomycin does not increase ATPase activity by altering membrane ion gradients nor directly interacting with the enzyme, but possibly through regulatory effectors or covalent modification of the enzyme.

Syringomycin is a peptide toxin produced by the plant pathogen, *Pseudomonas syringae* pv *syringae* (12). Although it has been purified, the complete structure of syringomycin has not been determined (11, 14). The toxin is not specific for a particular host plant but is a major virulence factor in several diseases caused by various ecotypes of *P. syringae* pv *syringae* (10). In addition, a number of fungi are highly sensitive to syringomycin (8, 18).

Several studies indicate that syringomycin has a primary effect on fungal and plant cell plasma membranes (2, 16, 21, 22). The toxin was localized at the plasma membrane of fungal and plant cells by autoradiographic and immunochemical methods (12, 16), and alterations in fungal plasma membrane functions with syringomycin addition have been observed (21). Another study on maize shoots, however, has shown that mitochondria could be additional target sites for the toxin (19).

We have observed that syringomycin at concentrations between 0.2 and 3 μM quickly hyperpolarized the membrane potential and increased the cytoplasmic pH of the yeasts *Rhodotorula pilimanae* and *Saccharomyces cerevisiae* (21, 22). Similar levels of the toxin inhibited the growth of these organisms. Administration of syringomycin to preparations of the *R. pilimanae* plasma membrane stimulated the vanadate-sensitive ATPase activity. From these studies, we concluded that syringomycin has a primary effect on yeast plasma membrane transport functions, which include an alteration of the ATPase activity.

Results of experiments described in this report show that syringomycin stimulates the ATPase activity of plant plasma membranes similar to the effect observed with fungi. We used red beet storage tissue in these studies because the properties and function of its plasma membrane ATPase have been sufficiently described (4–6, 15).

MATERIALS AND METHODS

Purification of Syringomycin. Syringomycin was extracted from liquid cultures of *Pseudomonas syringae* pv *syringae* strain B301D which was grown to stationary phase (21). The cultures were extracted with an equal volume of acidified acetone (0.046 N HCl in acetone), and the mixture was centrifuged at 5000*g* for 10 min. The supernatant was collected and concentrated to 1/10 volume at 45° to 50°C with a rotary evaporator. Acetone was added to a concentration of 60% (v/v) and the suspension was gently stirred overnight at 4°C. The suspension was centrifuged, and the supernatant was removed and concentrated as before and then diluted to 1 L with 0.1% TFA.[2] The TFA solution was applied to an Amberlite XAD-2 (20- to 50-mesh) column (3 × 25 cm). The column was equilibrated with 0.1% TFA before use. Syringomycin was eluted with a nonlinear gradient of 0.1% TFA and 0.1% TFA in 2-propanol with the latter increasing in proportion from 0 to 20% (elapsed time 20 min), maintained at 20% (elapsed time 140 min), then from 20 to 70% (elapsed time 240 min), maintained at 70% (elapsed time 270 min), then from 70 to 100% (elapsed time 300 min), and finally maintained at 100% for 60 min. The eluant was monitored at 435 nm. The peak syringomycin fractions (as determined by bioassay [18] with *R. pilimanae* [21]) were pooled, and the 2-propanol was removed with the rotary evaporator. The concentrate was then lyophilized. The dried sample was dissolved in water:acetone (40:60 v/v) and subjected to high performance reverse-phase liquid chromatography on a 1 × 25 cm, C18 column (5 μ, Alltech Associates, Inc.) with a 2-propanol gradient (0–100% in 35 min, 0.1% TFA-0.1% TFA in 2-propanol). The syringomycin peak was identified by bioassay and collected. For

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[2] Abbreviations: TFA, trifluoroacetic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

storage, the 2-propanol was removed by rotary evaporation and the sample lyophilized. This procedure yielded a syringomycin preparation (Fig. 1) with a specific activity of 35 to 50 units/μg. A unit of activity was defined as the amount of toxin which completely inhibited the growth of *R. pilimanae* on potato-dextrose agar plates where a 10 μL droplet was applied.

Fractionation of Red Beet Storage Tissue. Red beet (*Beta vulgaris* L.) storage tissue with fresh intact leaves was purchased from a local grocery store and used immediately. The microsomal fraction was obtained and extracted with KI following the procedure of Briskin and Poole (4). The KI-extracted microsomal material was suspended in suspension buffer containing 250 mM sucrose, 1 mM Tris-Mes (pH 7.2), and 1 mM DTE. It was then centrifuged on linear sucrose density gradients (15–45% w/w) at 120,000*g* for 3 h and 4°C in a Beckman SW 41 rotor. The sucrose solutions were buffered with 1 mM Tris-Mes (pH 7.2) and contained 1 mM DTE.

Preparation of Plasma Membranes. For the large-scale preparation of plasma membranes, red beets stored between 1 and 6 months in moist vermiculite at 4°C were used. KI-extracted suspensions were prepared as described above. The sample was applied to step density gradients containing 5.5 mL of 25% (w/w) sucrose and 6 mL of 34% (w/w) sucrose (each in 1 mM Tris-Mes [pH 7.2] and 1 mM DTE) in Beckman SW 41 rotor tubes. The gradients were centrifuged at 107,000*g* for 2 h at 4°C. The plasma membranes sedimenting at the sucrose interface were recovered and used immediately or frozen in liquid N_2 and stored at −70°C.

Deoxycholate and Zwittergent 3-14 Treatment of Plasma Membranes. Plasma membrane preparations were diluted to 2 mg protein per mL. The deoxycholate treatment was patterned after the method of Briskin and Leonard (3). The membranes were treated with an equal volume of 4 mM EDTA, 10 mM ATP (disodium salt), 200 mM KCl, 50 mM Tris-HCl (pH 7.5), 1 mM DTE, and 0.2% (w/v) sodium deoxycholate. The solution was added to the membranes slowly while stirring at ice temperature, and the mixture was then incubated for 20 min on ice. The mixture was centrifuged at 200,000*g* for 30 min at 4°C in a Beckman 70.1 Ti rotor, and the pellet (deoxycholate-treated plasma membrane) was dissolved in suspension buffer. For reconstitution, the pellet was suspended in a proportionate amount of the 200,000*g* supernatant material.

For Zwittergent 3-14 treatment, the suspended deoxycholate-treated membrane material was adjusted to 2 mg of protein per mL. A solution containing 40% (v/v) glycerol and 0.2% (w/v) Zwittergent 3-14 in suspension buffer was added slowly while stirring on ice. After incubation for 20 min, the mixture was centrifuged at 200,000*g* for 30 min in the 70.1 Ti rotor. The supernatant containing solubilized ATPase activity was recovered.

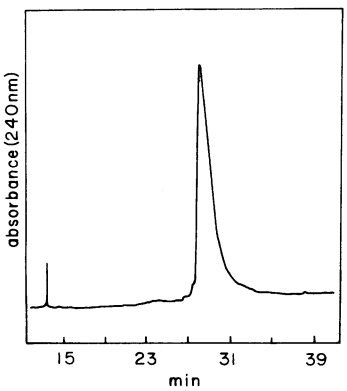

FIG. 1. HPLC absorbance profile of purified syringomycin (2.6 mg). Chromatography was done on a C-18 column (Alltech, 5 μ) with a 35 min 2-propanol gradient.

Both the deoxycholate and Zwittergent 3-14 treated samples were frozen in liquid N_2 and stored at −70°C.

ATPase Assay. ATPase activities were determined as described by Briskin and Poole (4). The standard assay was done in 1 ml volumes containing 3 mM Na_2ATP, 3 mM $MgSO_4$, 50 mM KCl, 30 mM Tris-Mes (pH 6.5), and enzyme. The reaction was initiated with the addition of Na_2ATP and incubated for 20 min at 38°C. Pi released was measured by the method of Ames (1). Na vanadate (0.5 mM), Na azide (10 mM), K nitrate (0.5 M), and syringomycin (2 mg/ml) were each prepared in distilled H_2O and added immediately before starting the reaction with ATP. The KCl concentration was varied in the experiments that concerned the effects of KCl on ATPase activity. For the experiments on the effects of ionophores on ATPase activity, valinomycin, nigericin, gramicidin, and FCCP were added as freshly prepared ethanolic solutions (2 mg/ml). The ionophores were added to the reaction mixture containing membranes, and the mixture was incubated for 2 min at 25°C before the addition of Na_2ATP and syringomycin. The ionophores were obtained from Sigma Chemical Co. Except for Figure 2, enzyme specific activities are expressed as μmol Pi released/mg protein·h.

Protein Assay. Protein levels were determined by the method of Markwell *et al.* (13).

RESULTS

Effects of Syringomycin on ATPase Activities of Cell Fractions. Microsome preparations obtained from red beet storage tissue homogenates were fractionated by sucrose density gradient centrifugation (15–45% [w/w] sucrose). The fractions were then analyzed for ATPase activities corresponding to the tonoplast, mitochondrial, and plasma membranes (Fig. 2). These activities were distinguished by their specific sensitivities to nitrate, azide, and vanadate, respectively (4, 9, 15). The three fractions were resolved: the tonoplast, plasma, and mitochondrial membranes sedimented at approximately 30% (w/w) sucrose, 40% (w/w) sucrose, and the bottom of the gradient, respectively. The activity found at the bottom of the sucrose gradient represented a small portion of the total mitochondrial activity of the homogenate. The bulk of this activity occurred in a pellet fraction following an earlier 13,000*g* differential centrifugation of the cell homogenate (data not shown; 4).

The effects of syringomycin on the ATPase activities of the various sucrose gradient fractions were determined (Fig. 2). The activities associated with the plasma membrane were preferentially stimulated. The nitrate-sensitive, tonoplast activity was also stimulated, but the stimulation was 3.3-fold lower than with the plasma membrane activity.

Characterization of the Stimulation of the Plasma Membrane ATPase by Syringomycin. With plasma membranes purified from KI-extracted microsomes, optimal (1.7- to 2.1-fold) stimulation was routinely achieved with 10 to 50 μg syringomycin/25 μg protein·ml (Fig. 3). This amount of syringomycin was equivalent to 500 to 2500 units. Syringomycin stimulation could be observed at levels as low as 2.5 μg/25 μg protein·ml. At levels higher than 200 μg, the activity was inhibited. Plasma membrane preparations that had been frozen in liquid N_2, stored for 2 h at −70°C, and then thawed still retained an activity which was stimulated by syringomycin, but the stimulation was lower (Fig. 3).

Treatment of the plasma membranes with 0.1% (w/v) deoxycholate followed by centrifugation resulted in a preparation (membrane pellet) with ATPase activity, but the activity was not stimulated by syringomycin (Fig. 4). Reconstitution of the supernatant material (recovered after deoxycholate treatment) with the treated membranes did not restore the stimulatory effect.

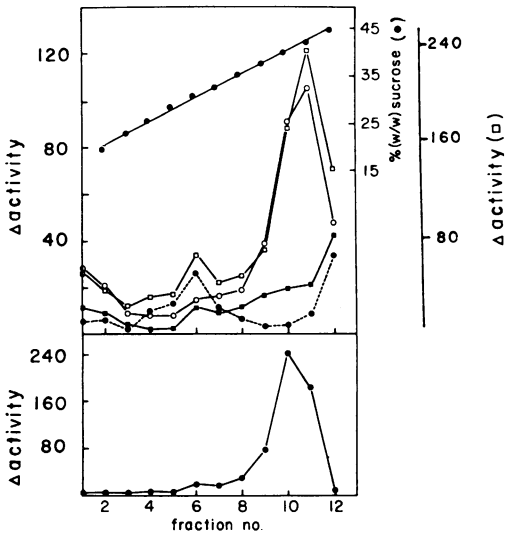

FIG. 2. Sucrose density gradient profiles of inhibitor-sensitive and syringomycin-stimulated ATPase activities after centrifugation of microsomes from fresh red beet storage tissue. Upper panel: Gradient fractions were treated with 50 mM K nitrate (●- - -●), 1 mM Na azide (■), or 50 μM Na vanadate (○) before assaying ATPase activities. The inhibitor-sensitive activities (△ activity) were obtained by subtracting values determined with inhibitor from control values obtained without treatment. The control activities are shown separately (□). Fraction 12 was the pellet at the tube bottom. Bottom panel: The same gradient fractions were treated with syringomycin (27 μg) and assayed for ATPase. The activities (△ activity) were calculated by subtracting values obtained without syringomycin from values obtained with syringomycin. Units of activity are nmol phosphate/mL·20 min.

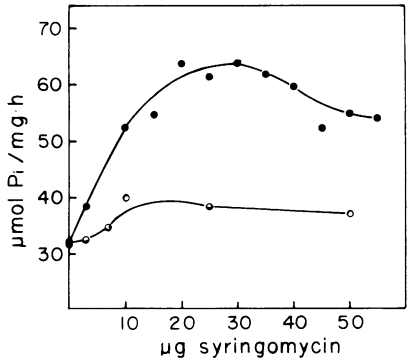

FIG. 3. Effect of syringomycin on the ATPase activity of KI-extracted plasma membranes of red beet storage tissue. The enzyme was assayed with either fresh (●) or −70°C stored (○) plasma membrane preparations.

The ATPase was solubilized with Zwittergent 3-14 (6), and syringomycin was then added. Syringomycin at levels between 2.5 and 10 μg/25 μg protein did not stimulate the enzyme activity (Fig. 4).

As previously observed (4), increasing the KCl concentration in the assay mixture slightly increased the ATPase activities (Fig. 5). Maximum activities were achieved with 20 mM KCl and higher. Syringomycin stimulation of the enzyme activity was similar at all KCl concentrations examined (Fig. 5).

Effects of Ionophores on ATPase Activity and Stimulation by Syringomycin. The increased ATPase activity associated with syringomycin could be due to a reduced electrochemical potential across the plasma membrane caused by syringomycin treatment. If so, tightly sealed plasma membrane vesicles with the ATPase catalytic site on the external surface are required, and these should show an increased ATPase activity with the addition of ionophores (20). To determine whether the plasma membranes used in these studies were tightly sealed, the membrane preparations were preincubated with one of several ionophores and the ATPase activities measured (Fig. 6). Valinomycin (1–10 μM), nigericin (1–5 μM), gramicidin (1–10 μM), or FCCP (2.5–10 μM) did not enhance the activity. When syringomycin (10 μg) was added to membranes that were individually pretreated with these ionophores, an increased enzyme activity was observed in each case (Fig. 6).

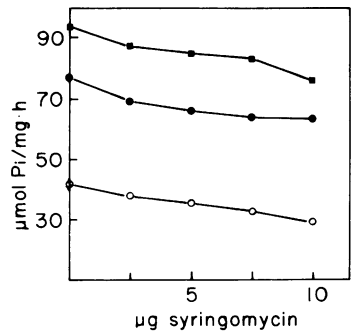

FIG. 4. Effect of syringomycin on the plasma membrane ATPase treated with 0.1% (w/v) deoxycholate (●), treated with deoxycholate and reconstituted with the supernatant fraction obtained after centrifugation (○), or solubilized with Zwittergent 3-14 (■).

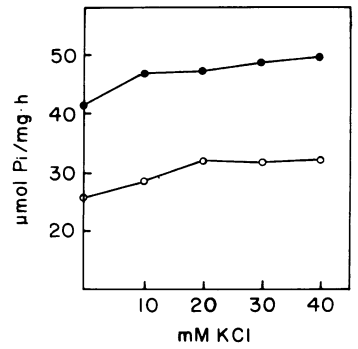

FIG. 5. Effect of KCl on the plasma membrane ATPase activity with (●) or without (○) syringomycin. Syringomycin and protein levels were 10 μg and 25 μg/ml, respectively.

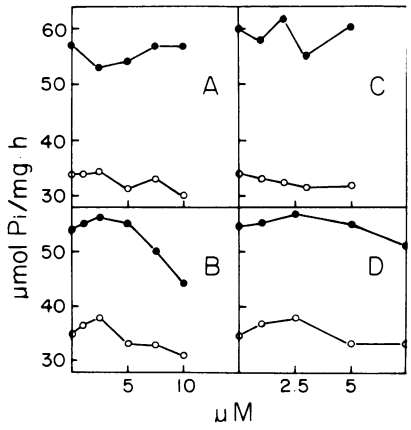

FIG. 6. Effect of ionophores on the plasma membrane ATPase activity assayed with (●) or without (○) syringomycin (10 μg/ml). The membranes (25 μg protein/ml) were briefly (2 min) pretreated with the indicated amounts of valinomycin (panel A), FCCP (panel B), nigericin (panel C), or gramicidin (panel D) before the ATPase assay.

DISCUSSION

Syringomycin stimulated the vanadate-sensitive ATPase associated with the plasma membrane of red beet storage tissue. This enzyme is believed to use ATP to pump protons across the plasma membrane (20). The tonoplast ATPase activity increased only slightly with amounts of syringomycin which consistently increased the plasma membrane ATPase activity 2-fold. Syringomycin apparently did not stimulate the mitochondrial ATPase. The effect on the plasma membrane ATPase is similar to that observed with the plasma membrane fraction of the yeast, *R. pilimanae* (21).

In addition to its sensitivity to vanadate, several other observations demonstrate that the proton-pump plasma membrane ATPase was affected by syringomycin. The catalytic specific activities measured (30–40 μmol Pi/mg protein · h) were the same as determined by others for the red beet enzyme (4). The activity was characteristically stimulated by KCl (4). The enzyme was enriched by treatment with 0.1% (w/v) deoxycholate and solubilized by 0.1% (w/v) Zwittergent 3-14 with no loss of activity (6). Finally, the density of the plasma membranes containing this activity decreased with cold storage of the red beet tissue (17). The syringomycin-stimulated ATPase activity was located at 40% (w/w) sucrose (ρ = 1.18 g/ml, 4°C) after sucrose density gradient centrifugation when fresh tissue was used (Fig. 2), and it was at the 25/34% (w/w) sucrose interface (ρ = 1.15 g/ml) with 1- to 6-month-old tissue (Fig. 3). These density values are similar to those reported by Poole *et al.* (7) for the plasma membrane-bound, vanadate-sensitive ATPase. ATPase stimulation by syringomycin was the same (about 2-fold) regardless of whether the plasma membrane was obtained from fresh or dormant red beet storage tissue.

Syringomycin did not appear to activate the plasma membrane ATPase by collapsing an electrochemical potential across the membrane as do certain ionophores (20). The membrane preparations were probably not sealed since they did not respond to ionophore addition (Fig. 6). Despite the apparent leakiness of the membranes, syringomycin stimulated the enzyme activity, even in the presence of ionophores. We conclude that syringomycin does not act like any of the ionophores examined, and its action on the ATPase does not involve indirect responses to altered transmembrane ion gradients.

The toxin might work directly on the enzyme. However, partially purified enzyme solubilized with Zwittergent 3-14 was not affected by syringomycin (Fig. 4). Syringomycin might also act through one or more regulatory effectors or covalent modification of the ATPase. We are currently investigating the possibility that syringomycin may alter a protein kinase-mediated phosphorylation of this enzyme. Such a mechanism is consistent with the observation that 0.1% (w/v) deoxycholate eliminates the ability of syringomycin to stimulate ATPase activity (Fig. 4). Briskin and colleagues (3, 7) have shown that deoxycholate reduces a membrane-bound protein kinase activity associated with the plasma membrane ATPases of corn roots and sugarbeet.

The increased ATPase activity with syringomycin could be due to detergent lysis fo the plasma membrane to allow access of ATP to the enzyme. This would require that a fraction of the ATPase catalytic sites be located on the inside surfaces of membrane vesicles which, despite being leaky to small ions, were impermeable to ATP. Although this possibility cannot be ruled out, there are arguments against it. (a) At the syringomycin concentrations used (2.5–50 μg/ml), detergent action is unlikely. For example, red beet tonoplast vesicles maintain an ATP-dependent pH gradient even with syringomycin at 100 μg/ml; the gradient then collapses when gramicidin is added (L Zhang, JY Takemoto, unpublished data). Thus, syringomycin at higher concentrations than used in the present study does not permeabilize this membrane even to protons. (b) In yeast cells, syringomycin at concentrations up to 6 μg/ml causes an increase of the membrane potential which cannot be explained by detergent lysis (21, 22). Despite this, syringomycin could act as a detergent at high concentrations. Whether its role in plant disease is related to its possible detergent action needs further investigation.

Syringomycin is produced extracellularly by *P. syringae* pv syringae and *in vivo* would presumably elicit its effects on the host plasma membrane at a site on the extracellular surface. However, the isolated membranes used in these studies had the opposite cytoplasmic surface exposed so that the ATPase was accessible to ATP. Two possibilities to consider for the side of the membrane on which syringomycin acts when added to the isolated membranes are: (a) syringomycin is capable of binding and stimulating the ATPase activity from either side of the membrane, or (b) the isolated membranes were sufficiently leaky to allow access of syringomycin or ATP to their respective binding sites on the extracellular or cytoplasmic surfaces. Knowledge about the molecular binding site for syringomycin and its membrane topography is needed to determine these possibilities.

Our results appear to differ from a report that mitochondria of corn shoots were affected by syringomycin (19). It was suggested that syringomycin acted as an uncoupler and thus stimulated the mitochondrial ATPase activity. We observed no apparent stimulation of the ATPase associated with mitochondria in red beet (Fig. 2). However, it is possible that the mitochondrial membranes in our preparations were not sealed, in which case an uncoupler would show no effect. The discrepancy might also reflect differences in plant species or tissue. These points warrant further investigation.

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