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Syringomycin stimulation of potassium efflux by yeast cells

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The phytotoxin, syringomycin, produced by *Pseudomonas syringae* pv. *syringae* caused the cellular efflux of K^+ in cell suspensions of *Rhodotorula pilimanae* and *Saccharomyces cerevisiae*. K^+ levels in the suspension media increased 3–10-fold within 2 min after adding syringomycin (1.5 μg/ml). As shown previously, syringomycin also stimulated the uptake of tetraphenylphosphonium (TPP) ions 10-fold. Although valinomycin (10 μM) and nigericin (10 μM) inhibited and *N,N'*-dicyclohexylcarbodiimide (DCCD) (50 μg/ml) stimulated TPP uptake, these ionophores had no effect on K^+ efflux. However, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) induced K^+ efflux and inhibited TPP uptake. We conclude that syringomycin causes K^+ efflux and that this effect is independent of the toxin's action on the DCCD-sensitive H^+-ATPase. Also syringomycin is not acting as a K^+ ionophore such as valinomycin or nigericin, nor does its action resemble that of the protonophore CCCP.

Introduction

Syringomycin is a phytotoxin produced by the bacterium *Pseudomonas syringae* pv. *syringae* and is a major virulence factor in a number of plant diseases caused by this bacterium [1]. Certain fungi are also sensitive to this toxin [2]. It is a cyclic lipopeptide with a M_r of 1224 [3]. Plasma membranes are proposed as the target of syringomycin [1,4,5], but the mechanism of action on this membrane is not known in detail. In previous reports [4–6], we demonstrated that in yeast cells syringomycin hyperpolarizes the plasma membrane, causes intra- and extracellular pH changes, and causes an efflux of K^+. Syringomycin also stimulates the plasma membrane H^+-pump ATPase of *Rhodotorula pilimanae* and red beet storage tissue [5,7]. These effects do not depend on mitochondrial function [4] and may be mediated by protein phosphorylation [8].

The nature of the K^+ efflux induced by syringomycin is unknown. One possibility is that the toxin acts like a K^+ ionophore allowing this ion, which occurs at high intracellular concentration (> 50 mM), to diffuse outward. Also, the relationship of the K^+ efflux of the stimulation of the H^+-pump ATPase and resultant pH changes is unknown. In this report, we characterize the K^+ efflux caused by syringomycin in both *R. pilimanae* and *Saccharomyces cerevisiae*. We show that syringomycin does not act like two well-known K^+ ionophores, valinomycin and nigericin to cause K^+ efflux. Also, this effect is independent of the stimulatory effect of syringomycin on the H^+-pump ATPase activity.

Materials and Methods

Organisms and growth. Methods for growing and harvesting *R. pilimanae* (ATCC 26423) and *S. cerevisiae* (D273-10B/A1, *rho*$^+$ and *rho*^{0}) were described previously [4,5].

TPP uptake. Methods used to measure the cellular uptake of TPP were described previously [4].

K^+ measurement. K^+ efflux was observed as the increase of extracellular K^+ concentration at room temperature. Cell suspensions were made as described previously [4] in 2 mM Tris-Mes buffer (pH 6.5) and 0.1 M glucose. The stock solutions of ionophores were 4 mM CCCP, 10 mM valinomycin and 20 mM nigericin all in ethanol. They were added immediately before the measurements began. DCCD (5 mg/ml in ethanol) was added into cell suspensions 10 min before the measurements. Ethanol added alone at the same levels as with the ionophores had no effect on K^+ efflux. Syringomycin (2 mg/ml in water) was added immediately before the measurements. Except for the experiments with DCCD, measurements were started with the ad-

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; Mes, 4-morpholineethanesulfonic acid; TPP, tetraphenylphosphonium.

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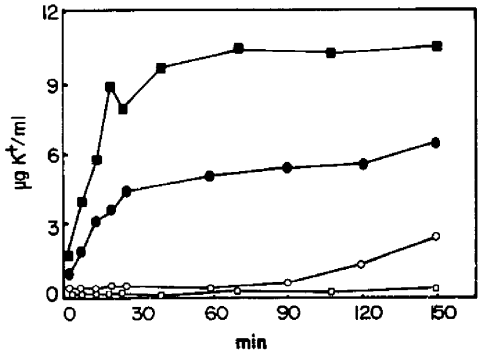

Fig. 1. The effect of syringomycin on K^+ efflux by *R. pilimanae* (●) and *S. cerevisiae (rho$^+$)* (■). Syringomycin (1.5 µg per ml) was added at zero time to cell suspensions. No syringomycin was added to controls for *R. pilimanae* (○) and *S. cerevisiae (rho$^+$)* (□).

dition of cells. At various times, 1 ml samples were withdrawn and centrifuged in an Eppendorf 5414 microcentrifuge for 30 s. The supernatant fluids (0.5 ml) were withdrawn and the K^+ concentrations of these were determined by atomic absorption spectroscopy (Instrumentation Laboratories, AA/AE Spectrophotometer 457).

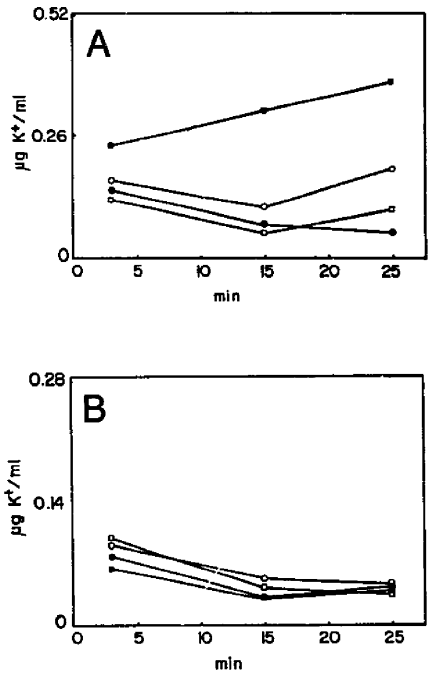

Fig. 2. Effects of ionophores on K^+ efflux by *R. pilimanae* (A) or *S. cerevisiae (rho$^+$)* (B). Either 10 µM CCCP (■), 10 µM nigericin (□), 10 µM valinomycin (●), or nothing (○) was added to the measuring medium.

Syringomycin. Syringomycin was provided by R.C. Bachmann (Utah State University) and purified as previously described [7]. The preparations used in this study had specific activities of 12800 units per mg [7].

All experiments reported were performed a minimum of three times and similar results were obtained. Data from representative single experiments are presented.

Results

Effect of syringomycin on cellular K^+ efflux

Without adding K^+ to the cell suspensions, syringomycin (1.5 µg per ml) stimulated K^+ efflux from cells of both *S. cerevisiae* and *R. pilimanae* (Fig. 1). Efflux was observed within 2 min after adding the cells which was the time needed to process the supernatant samples for the K^+ measurements. K^+ efflux continued to increase (3–10-fold) for 30 min before leveling. The same results were achieved with the petite *rho°* strain of *S. cerevisiae* (data not shown).

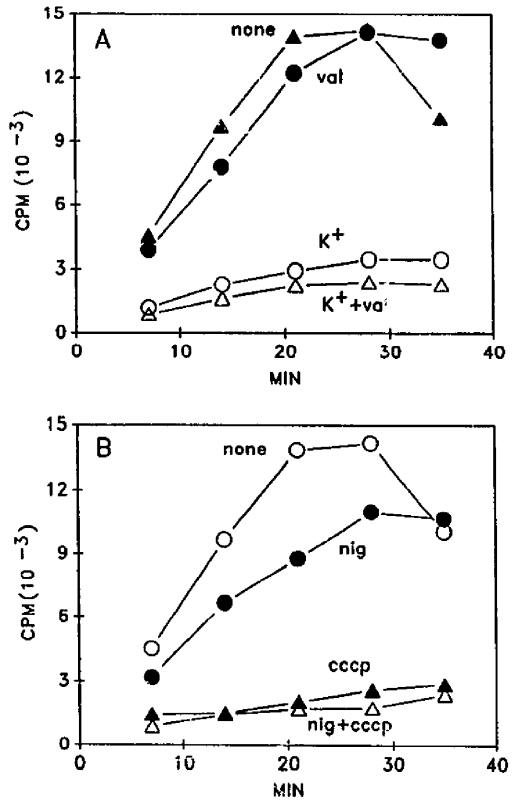

Fig. 3. Effects of valinomycin (A) and nigericin (B) on TPP uptake by *R. pilimanae.* In (A) either 10 µM vlainomycin (●), 0.1 M KCl (○), 10 µM valinomycin plus 0.1 M KCl (△), or nothing (▲), and in (B) 10 µM nigericin (●), 10 µM CCCP (▲), 10 µM nigericin plus 10 µM CCCP (△) or nothing (○) was added to the cell suspension medium.

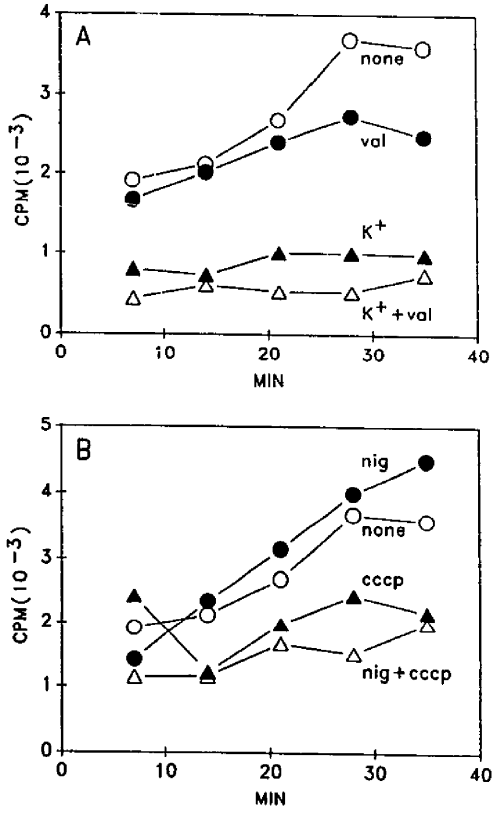

Fig. 4. Effects of valinomycin (A) and nigericin (B) on TPP uptake by *S. cerevisiae (rho°)*. The ionophore and KCl concentrations were the same as for Fig. 3.

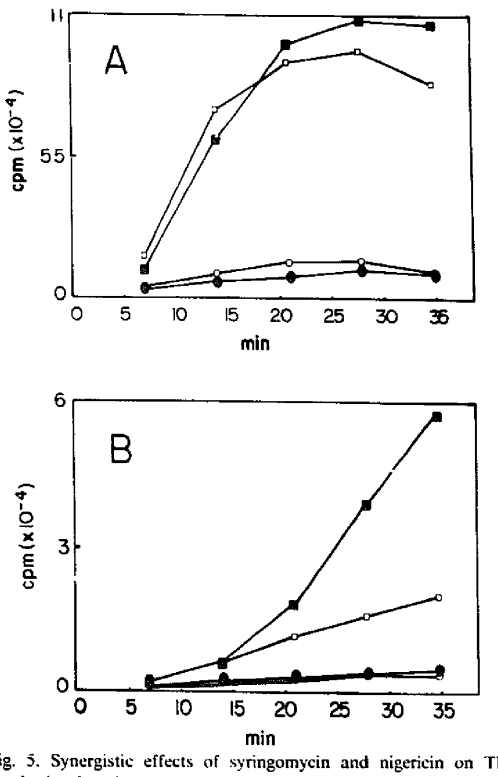

Fig. 5. Synergistic effects of syringomycin and nigericin on TPP uptake by *R. pilimanae* (A) and *S. cerevisiae (rho°)* (B). Either 1.5 μg/ml of syringomycin (□), 10 μM nigericin plus 1.5 μg/ml syringomycin (■), 10 μM nigericin (●), or nothing (○) was added.

Effects of K^+ ionophores

When the K^+ ionophores valinomycin (10 μM) or nigericin (10 μM) were added to cell suspensions of either yeast organism, no effects on net K^+ efflux were observed (Figs. 2A and 2B). Also, in contrast to syringomycin [4–6], valinomycin and nigericin did not increase the membrane electrical potentials (inside negative) of *R. pilimanae* and *S. cerevisiae* measured as the rate of TPP uptake (Figs. 3 and 4). To demonstrate that valinomycin and nigericin were active at the concentrations used, their effects on TPP uptake when added together with KCl and CCCP, respectively, were tested. As expected, valinomycin (10 μM) + KCl (0.1 M) greatly inhibited TPP uptake in both organisms (Figs. 3A and 4A). KCl at 0.1 M concentration also inhibited TPP uptake, but in combination with valinomycin the inhibition was slightly, but consistently, greater. Similarly, nigericin (10 μM) + CCCP (10 μM) consistently prevented TPP uptake to a slightly greater extent than CCCP alone (Figs. 3B and 4B). Thus, the two K^+ ionophores were active in these experiments.

The combined results show that syringomycin did not work like valinomycin or nigericin in causing K^+ efflux.

When syringomycin and nigericin were added together, the rate of TPP accumulation was greater than

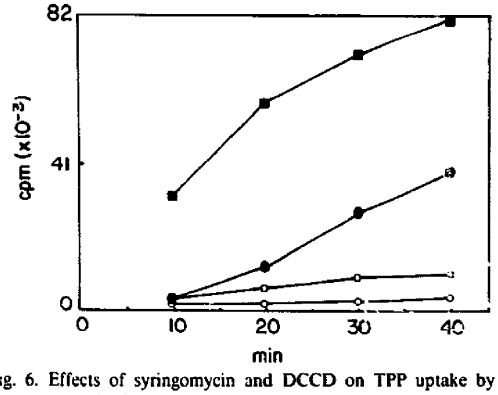

Fig. 6. Effects of syringomycin and DCCD on TPP uptake by *S. cerevisiae (rho°)*. Cells were preincubated with 50 μg/ml DCCD for 10 min and then either syringomycin (1.5 μg/ml) (■) or nothing (□) was added. In control experiments, cells were preincubated but with no DCCD, and either syringomycin (1.5 μg/ml) (●) or nothing (○) was added.

with syringomycin alone (Fig. 5). This synergistic effect was more pronounced with *S. cerevisiae* than with *R. pilimanae*.

Effects of DCCD

To investigate the relationship between K^+ efflux caused by syringomycin and its effect on the H^+-ATPase, experiments were conducted with DCCD and the *rho*° strain of *S. cerevisiae*. Because the *rho*° strain lacks functional mitochondria, DCCD primarily affects the plasma membrane H^+-ATPase of intact cells [9,10]. The rate of TPP uptake by DCCD (50 μg/ml)-treated cells was slightly higher than by untreated cells (Fig. 6). With the further addition of syringomycin, TPP uptake was enhanced so that after 10 min the level of TPP uptake was 10-fold higher than controls. Cells treated with syringomycin required 30 min to achieve this rate of uptake. The same results were observed with the wild-type strain of *S. cerevisiae* and when *rho*° cells were preincubated with diethylstilbesterol (50 μg/ml) (data not shown).

The influence of DCCD on K^+ efflux by *S. cerevisiae* and *R. pilimanae* was determined. In contrast to its effect on TPP uptake, DCCD had no effect on K^+ efflux (Fig. 7). Preincubating the cells with DCCD did not result in further enhancement of K^+ efflux induced with syringomycin addition.

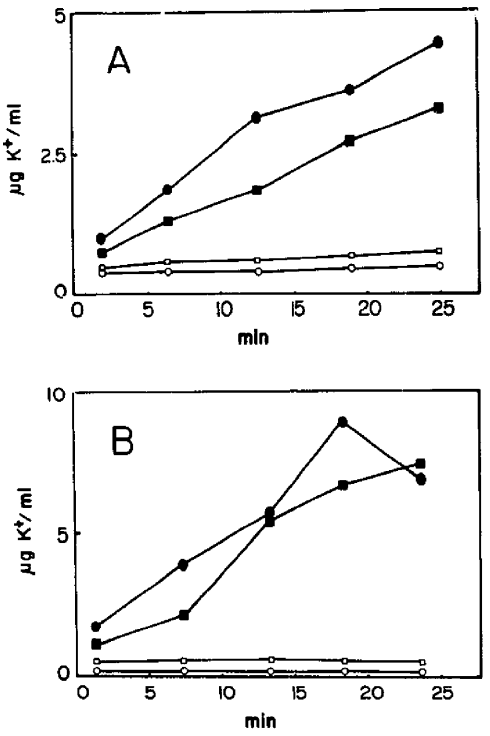

Fig. 7. Effects of syringomycin and DCCD on K^+ efflux by *R. pilimanae* (A) and *S. cerevisiae (rho⁺)* (B). The additions and symbols are the same as described for Fig. 6 except that no preincubation was performed.

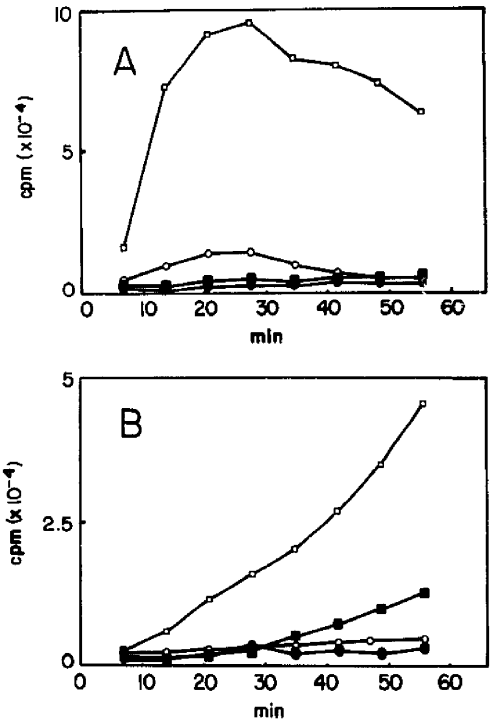

Fig. 8. Effects of syringomycin and CCCP on TPP uptake by *R. pilimanae* (A) and *S. cerevisiae (rho°)* (B). Either 10 μM CCCP (●), 1.5 μg/ml syringomycin (□), 1.5 μg syringomycin plus 10 μM CCCP (■), or nothing (○) was added to the medium.

Effects of the protonophore CCCP

CCCP caused K^+ efflux in *R. pilimanae* but not in *S. cerevisiae* (Fig. 2). No synergistic effect with this protonophore and syringomycin was observed. As mentioned above, CCCP (10 μM) did not stimulate TPP uptake in either organism. When syringomycin was added to cells pre-treated with CCCP, low rates of TPP uptake were observed (Fig. 8).

Discussion

The results show that syringomycin caused the cellular efflux of K^+. This effect was due to an alteration in the selective permeability to K^+, and was not due to the general disruption of the membrane. Since the toxin increased the electrical potential across the plasma membrane, it did not physically destroy this membrane. Because of this selectivity, we addressed the question of whether syringomycin acted as a K^+ ionophore. Our findings show that syringomycin did not behave like

two well-known K^+ ionophores, valinomycin and nigericin. Neither of these ionophores induced net K^+ efflux or stimulated TPP uptake as did syringomycin. Also, syringomycin appeared to act differently than CCCP, a protonophore which we have observed to cause K^+ efflux in yeast. As expected, CCCP decreased the plasma membrane electrical potential as measured by TPP uptake, whereas syringomycin hyperpolarized the membrane.

The K^+ efflux may be one component of the membrane hyperpolarization seen with administration of the toxin. Treatment of the *S. cerevisiae rho*^{0} strain with DCCD resulted in high rates of TPP uptake when syringomycin was added (Fig. 6). Since DCCD is known to inhibit the plasma membrane H^+-ATPase [9] and it did not abolish K^+ efflux, the increased net efflux of this ion may have contributed to the hyperpolarization in the absence of a H^+ gradient. The K^+ gradient is known to contribute to the electrochemical potential of the yeast plasma membrane [12,14].

K^+ effluxes are common in plant diseases and plant hypersensitivity responses [14,15]. The mechanisms leading to K^+ efflux in these cases, however, are not known. With yeasts, there are several reports that compounds such as DCCD, diethylstilbesterol, Dio-9 [16–18], trifluoperazine [19–21] and ethidium bromide [22] induce electrogenic K^+ effluxes. These reagents also hyperpolarize the yeast plasma membrane [13,17,18,20, 22,23]. In the work reported here, however, DCCD did not stimulate K^+ efflux (Fig. 7). Unlike syringomycin [5], some of these compounds are known to inhibit the plasma membrane H^+-ATPase, and their effects on K^+ efflux may reflect the activation of transport processes resulting from ATPase inhibition [17]. Direct effects of these reagents on an electrogenic K^+ pump or K^+/H^+ exchanger have been postulated [18,20], but the mechanisms are not known. Therefore, insight into how syringomycin may cause K^+ efflux is not provided by previous observations on the action of these compounds.

Overall, we conclude that syringomycin causes two major ion transport effects in the yeast membrane: H^+-ATPase stimulation and increased permeability to K^+. The question arises whether these two effects are coupled or are parallel secondary responses to a primary effect of syringomycin. Our observations with DCCD suggest that the K^+ efflux is independent of the effect on the H^+-ATPase. Also, syringomycin stimulates the H^+-ATPase in unsealed plasma membrane vesicles of red beet [7]. Therefore, the enhanced ATPase activity could not depend on a gradient of K^+. We conclude that the two effects are not directly coupled.

Acknowledgements

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