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THE RELATIONSHIP BETWEEN MEMBRANE ATPase ACTIVITY IN SUGARCANE AND HEAT-INDUCED RESISTANCE TO HELMINTHOSPOROSIDE

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Summary

1. Heating of susceptible sugarcane leaves (4 h at 35°C) renders them resistant, for 24 h, to the effects of helminthosporoside. Membrane ATPase activity is reduced by 50% as a result of the heat treatment. When the leaves again become susceptible (after 24 h), membrane ATPase activity is fully restored.

2. Inhibitors of membrane ATPase activity protect susceptible leaves from the effects of helminthosporoside (KF, EDTA, and octylguanidine).

3. Helminthosporoside activates (30%) membrane ATPase in microsomes from susceptible, but not heat-treated (resistant) leaves. Once heat-treated leaves again become susceptible, helminthosporoside activation of membrane ATPase activity resumes.

4. A plot of the production of helminthosporoside-induced symptoms, and membrane ATPase activity as a function of the reciprocal of the absolute temperature reveals that both have sharp breaks at 32°C.

5. Protoplasts of susceptible cane are rendered insensitive to the effects of the toxin in a medium deficient in K^+ and Mg^{2+}. When these ions are added, cell sensitivity to the toxin is restored. Since K^+ uptake in plants is mediated by membrane ATPase, a connection with this enzyme activity can be made to cell sensitivity to the toxin.

Introduction

There are numerous observations in the literature showing a detrimental effect of fungal and bacterial phytotoxins on the function of plant membranes [1,2]. In fact, several of these toxins selectively affect the plasma membrane [2,3]. It has been suggested that some toxins which are host specific, as well as membrane specific, may prove useful as tools or probes of normal membrane

form and function. Currently, with the availability of several purified and characterized host-specific toxins, such membrane studies seem feasible.

One system that may enable the study of protein-protein interaction on plant plasma membranes originates with the eyespot disease of sugarcane caused by the fungal pathogen *Helminthosporium sacchari.* The pathogen produces a host-specific toxin which is not only essential for its pathogenicity, but also for its host specificity. The toxin, known by the trivial name helminthosporoside, has a proposed structure of 2-hydroxycyclopropyl-α-D-galactopyranoside [4]. Thus far, of the 32 clones and mutants of sugarcane tested, only those that possess a functional toxin-binding protein are susceptible to the toxin and likewise the fungus [5,6]. The toxin-binding protein has multiple subunits, multiple binding sites for the toxin, and is localized in the plasma membrane [5,7]. A likely normal physiological role for the toxin-binding protein is in the transport of α-galactosides [8]. Evidence for the transport role of the protein resides in studies on the kinetics of binding α-galactosides by the purified protein and experiments on the uptake of labeled α-galactosides by sugarcane protoplasts [8]. The death of protoplasts brought on by toxin treatment cannot be readily correlated with the toxin-induced inhibition of α-galactoside uptake of the cell. On the other hand, one clue to the detrimental effect of the toxin on the cell is its activation of membrane ATPase acting via the toxin-binding protein [8]. This activation phenomenon may or may not be directly related to protoplast rupture, but the suggestion is a useful one considering the role that the membrane ATPase has in the regulation of K^+ concentration in the cell [9]. Therefore, this report is an expansion of the original observation of toxin activation of membrane ATPase [8] coupled with some data to suggest that this phenomenon may be a link in the process of toxin-induced cellular death. Pertinent to the present study are the observations of Byther and Steiner [10] who noted that sugarcane clones normally resistant to *H. sacchari* in the summer months become susceptible in the cooler seasons. Further, mild heat treatment of susceptible clones renders them temporarily resistant to helminthosporoside [11].

Experimental procedure

In vivo studies. Sections (15 cm long) of susceptible sugarcane leaves (clone 51 NG 97) were split down the midrib 3/4 of their length and 1/2 leaf placed upright in H_2O and the other half in a treatment solution. The leaves were treated for the indicated time period and then both half-leaves were administered 1 μl of H_2O containing 0.01 μmol of helminthosporoside on a small puncture wound made near the base of the leaf. After 1 h of incubation in a humidity chamber at 23°C, the leaves were removed and the length of the toxin-induced 'transparent or water-cleared' area was measured. This could effectively be accomplished by holding the leaf up to a light source to observe and measure the affected area of the leaf. This 'water clearing' symptom is to be distinguished from the more extensive red runner symptom that develops in the water-cleared area after 20—24 h [4].

In vivo heat treatment studies were conducted on 15-cm leaf sections that were placed at different temperatures in a Percival growth chamber under

500 ft-candles of fluorescent light. Toxin inoculations were made on each half-leaf and the measurement of symptoms was performed as described above. In all in vivo experiments at least six replicates were run and in the heat treatment studies, at least 12 replicates.

Membrane isolation and ATPase assay. The discontinuous sucrose gradient procedure of Leonard and Hodges [9], with some modification, was effectively used in isolating a microsomal fraction enriched with plasma membranes. The microsomal fraction at the interface of 34% and 45% sucrose layers peaked in ATPase (ATP phosphohydrolyase, EC 3.6.1.3) activity and possessed the greatest toxin-binding protein activity, both of which are indicative of the presence of plasma membrane vesicles [7,12]. Furthermore, when this fraction was fixed and stained with the chromic acid/phosphotungstate reagent of Roland et al. [13] and then examined by electron microscopy it revealed a mixture of vesicles with approximately 60% of them tentatively identified as plasma membranes. ATPase activity was measured under optimum conditions at 23°C in a 0.5 ml vol. containing 1.5 mM ATP (Tris salt), 1.5 mM $MgCl_2$, 50 mM KCl, and 50 mM Tris-HCl at pH 7.2. The reaction was started with the addition of 200—500 μg of membrane protein. The released inorganic phosphate over a heat-inactivated control was measured by the method of Lowry and Lopez [14]. Protein was measured according to Lowry et al. [15] using bovine serum albumin as a standard. The data are reported as μmol P_i/mg protein per h and the standard deviation of the mean calculated for any given determination is ±0.02. The standard deviation of the mean calculated between duplicate experiments was ±0.15. The equilibrium dialysis technique for assaying toxin-binding protein activity was used as previously described [5].

Protoplasts. The procedures used to prepare sugarcane protoplasts were similar to those described by Strobel and Hess [7] with the exceptions that loridine was not used, and the cellulase and macerozyme from Yakult Biochemicals were the 10X grade. Incubation times for tissues in the enzyme solution were 6—12 h. The recovered protoplasts (10^6/g tissue) were successively washed twice in 100 ml of 0.3 M sucrose containing 10 mM sodium citrate, pH 5.6.

Materials. Octylguanidine was a generous gift of Dr. Beatriz Gomez-Lepe of the Instituto de Biologica, Universidad Nacional Autonoma de Mexico.

Results

Toxin binding and heat treatment

The susceptible clone 51 NG 97 became completely resistant to the effects of helminthosporoside after immersion in H_2O at 50°C for 30 s, or when placed in a growth chamber at 35°C for 4 h as originally observed by Byther and Steiner [10]. Restoration of the leaves to complete susceptibility to the toxin occurred in 18—24 h at 23°C [11].

Toxin-binding activity was measured on the crude leaf membranes [5] of both a heat-treated (35°C, 4 h) and a control (23°C) set of leaves. The membranes from heat-treated leaves bound 1.7 nmol of helminthosporoside/mg protein, whereas the preparation from control leaves bound 1.6 nmol of

helminthosporoside/mg protein. Comparable results were obtained in preparations from leaves which were immersed in H_2O at 50°C for 30 s.

ATPase activity and heat treatment

Inasmuch as there was no detectable effect of heating on the binding activity of the toxin-binding protein, at 35°C, the involvement of other factors was considered. From a previous study [8], it was noted that helminthosporoside activates membrane ATPase. Thus, the involvement of ATPase as a heat-sensitive activity was examined in greater detail as it related to the mechanism of action of the toxin.

Byther and Steiner [10] showed that heat-treated susceptible leaves did not regain susceptibility when they were administered either actinomycin D or placed in an N_2 atmosphere. Table I shows that membrane ATPase lost at least 50% of its activity upon heating the leaves, from which the membranes were prepared, at 35°C for 4 h. Furthermore, when heated leaves were placed in a solution of 10 μg/ml actinomycin D for 24 h or if placed in an N_2 atmosphere for 24 h, the ATPase activity remained suppressed. These results showed that the membrane ATPase is heat sensitive and the same treatments that maintained resistance in heat-treated leaves, i.e. actinomycin D or N_2, also kept the ATPase activity suppressed. This interesting correlation led to the next logical step of determining the variation of symptom expression and ATPase activity as a function of temperature.

Leaves of clone 51 NG 97 were heated for 4 h in the growth chamber at a constant temperature in a range from 28 to 39°C. The leaves varied in their response to the toxin as a function of the temperature of incubation. When symptom expression was plotted as a function of the reciprocal of the absolute temperature, a sharp break in the plot occurred at 32°C ± 1°C (Fig. 1). The

TABLE I

THE EFFECT OF HEATING-SUSCEPTIBLE SUGARCANE FOLLOWED BY TREATMENT WITH VARIOUS INHIBITORS OF MEMBRANE ATPase ACTIVITY

Sugarcane clone 51 NG 97 was used. The heat treatments (35°C, 4 h) indicated were applied to 12 g of leaves, following which the leaves were exposed to other treatments prior to extraction and preparation of microsomes for membrane ATPase activity measurements. The final state of the leaves, relative to susceptibility or resistance to 1 μl of 0.01 μmol of helminthosporoside, is indicated. Standard deviation of the mean calculated for any given determination is ±0.02 and between duplicate experiments it was ±0.15.

Treatment	Membrane ATPase activity (μmol P_i/mg protein per h)
Control (not heated), susceptible	1.2
Heated (leaves heated 35°C, 4 h), resistant	0.6
Heated (leaves heated 35°C, 4 h, then exposed to 10 μg/ml actinomycin D for 24 h), resistant	0.6
Heated (leaves heated 35°C, 4 h, then exposed to a pure N_2 atmosphere for 24 h), resistant	0.5
Heated (leaves heated 35°C, 4 h, then exposed to 23°C, normal atmosphere for 24 h) susceptible	1.2

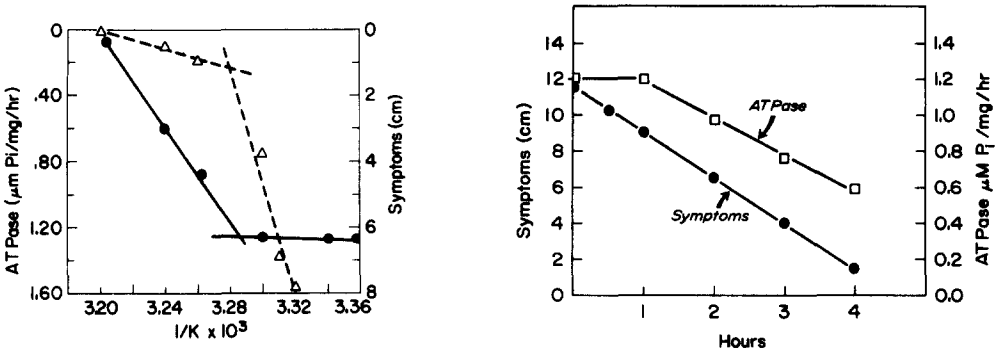

Fig. 1. Plots of symptom expression and membrane ATPase activity as a function of the reciprocal of the absolute temperature. Each datum point for symptom expression (△———△) was an average of 12 determinations. Each datum point for membrane ATPase activity (· · · · ·) was an average of at least two determinations ±0.15. Leaves were placed at each temperature for 4 h and the determination of membrane ATPase activity made on some leaves, and on others symptom expression was measured after inoculation with 1 μl of 0.01 mol helminthosporoside.

Fig. 2. Membrane ATPase activity and symptom expression as a function of time of exposure to 35°C. Membrane ATPase activity and symptom expression were measured on separate sets of leaves for each determination. The data for both determinations are presented as the line of best fit by least-squares analysis. Each datum point for symptom expression (· · · · ·) represents an average of 12 determinations, and each datum point for membrane ATPase activity (□———□) represents an average of two determinations from different sets of heat-treated leaves.

microsomal fraction of leaves treated under the same temperature conditions were assayed for membrane ATPase activity. The data from these assays were plotted in a comparable manner and a sharp break also occurred in the plot at 32°C ± 0.8°C (Fig. 1). Close examination of Fig. 1 reveals that at 30°C (3.30) there is a 50% reduction in symptom expression before any effect is noted in membrane ATPase activity. Since symptom expression is a complex situation involving toxin movement, toxin binding, water and ion movement plus other physiological effects, it is not surprising that at any given temperature the degree of symptom expression is not correlated with membrane ATPase activity. Nevertheless, since membrane ATPase activity and symptom expression share the same critical inflection at 32°C, the implication is that the two phenomena may be related.

Susceptibile leaves were heated at 35°C for times varying from 0 to 4 h. In comparable experiments, membrane ATPase activity was measured in these heat-treated tissues. The degree of symptom expression and membrane ATPase activity were plotted as a function of time of exposure at 35°C. The results (Fig. 2) show a linear reduction in the degree of symptom expression as well as a linear reduction in detectable membrane ATPase activity. The plots of both relationships have similar slopes between the 1 h and the 4 h heat treatments.

Helminthosporoside exhibits an activation effect on membrane ATPase [8]. If the membrane ATPase were playing some role in the toxin-mediated tissue response, then one might suspect that the toxin activation of ATPase would also be affected by the heat treatment. Susceptible leaves were heat treated at 35°C for 4 h and then assayed for membrane ATPase activity. The activity was 50% of the non-treated control (Table II). Further, the membrane ATPase

TABLE II

EFFECTS OF HEMINTHOSPHOROSIDE AND HEAT TREATMENT ON THE ATPase ACTIVITY OF SUSCEPTIBLE SUGARCANE LEAF MEMBRANES

All reaction mixtures contained the optimum amounts of reactants for membrane ATPase activity, see Experimental procedure.

Treatment	Membrane ATPase activity (μmol P_i/mg per h)
Full reaction mixture (control)	1.2
Full reaction mixture plus 0.3 mM helminthosporoside	1.6
Heat treatment *	0.6
Heat treatment plus 0.3 mM helminthosporoside	0.6
Heat treatment followed by 24 h at 23°C	1.2
Heat treatment followed by 24 h at 23°C plus 0.3 mM helminthosporoside	1.6

* Leaves were heated at 35°C for 4 h prior to extraction of microsomes for membrane ATPase assay.

activity that was present in the microsomal fraction from the heat-treated leaves was not activated by helminthosporoside. When allowed to stand at 23°C for 24 h, the heat-treated leaves again became susceptible to helminthosporoside. Likewise, the membrane ATPase was again activated by helminthosporoside (Table II).

Effects of inhibitors

Half-leaves were treated with various concentrations of inhibitors for 24 h and then treated with the toxin. Concentrations for the inhibitors that were effective in precluding symptom production were also tested for their ability to inhibit membrane ATPase activity. At 100 mM, KF completely inhibited symptom production and also membrane ATPase activity (Table III). EDTA at 10 mM also totally precluded symptom production. EDTA, because of its divalent cation-chelating properties was anticipated to be an effective inhibitor

TABLE III

THE EFFECT OF VARIOUS INHIBITORS ON SYMPTOM DEVELOPMENT IN THE HALF-LEAF BIOASSAY TEST ON SUSCEPTIBLE SUGARCANE AND ON ATPase ACTIVITY

Runner length was measured 1 h after toxin application. All treatments were for 24 h. At least six replicates were run for each determination. The development of runners between the treatment and control half-leaves were all statistically different at the 0.01 level according to the Student's *t*-test. Calculations were made as: (treatment/control) X 100 — 100. All assays for membrane ATPase were run under standard conditions, using the levels of inhibitors indicated.

Treatment	Percent change in runner length inhibition	Membrane ATPase activity (μmol P_i/mg per h)
None	0	1.2
KF		
100 mM	100	0
20 mM	68	0.7
EDTA, 10 mM (pH 7.0)	100	0
Octylguanidine		
10 mM	100	0.6
1 mM	90	0.6

since Mg^{2+} is required for maximal ATPase activity [9]. Octylguanidine is a specific inhibitor of membrane-bound ATPase of yeast and mammalian cells [6,17—20] and more recently it has been shown to inhibit K^+ transport in plant roots [21]. At both 1 and 10 mM, it was extremely effective in preventing toxin-induced symptom expression. Furthermore, at that concentration, it reduced the membrane ATPase activity by 50%. Sodium metavanadate, is an inhibitor of $(Na^+ + K^+)$-ATPases from a number of sources [22,23]. At 1 mM it had no protective effect on the leaves against the toxin and membrane ATPase activity was unaffected.

Protoplast experiments

Washed protoplasts of clone 51 NG 97 were administered 1 mM helminthosporoside in the presence and absence of 1.5 mM $MgCl_2$ and 10 mM KCl. Controls, not treated with helminthosporoside, were also set up and 2 h after toxin treatment, at least 25% of the protoplasts (over the controls) were dead in the solution containing ions plus toxin, whereas only 5% had died in the solution with the toxin alone. After 4 h, these values rose to 80% and 17%, respectively. When 1.5 mM $MgCl_2$ and 10 mM KCl were added to the protoplasts in the solution with toxin alone, up to 95% of the protoplasts died within 2 h. The salts had no adverse effect on the protoplasts in the control.

Discussion

The protective effect of heat treatment on susceptible sugarcane varieties to helminthosporoside is not due to the denaturation of the toxin-binding protein. This is evidenced by the fact that heat treatment of the susceptible clone 51 NG 97 at either 50°C for 20 s or 35°C for 4 h, which renders the plant resistant to the effects of helminthosporoside, did not cause a decrease in the binding of the toxin to the toxin-binding protein. Therefore, it is apparent that the detrimental effects of the toxin on the susceptible plant cell must be mediated via more than a one step process of the binding of the toxin to a receptor site (the toxin-binding protein). Yet it appears that toxin binding to the binding protein is the critical first step in toxin-induced cell death, as witnessed by the inhibitory effects of α-galactosides on toxin-induced symptoms [5], by mutation studies on susceptible cane varieties [6], and by the actual transfer of the binding protein to resistant cane and tobacco protoplasts, rendering them susceptible to the toxin [24].

Our previous report [8] on membrane ATPase activity in sugarcane indicated much lower specific enzyme activities. This discrepancy with the current report may be related to the difficulties commonly encountered in accurately determining membrane protein content on intact microsomes. Nevertheless, the membrane ATPase activities in this report are still relatively low when compared to those reported in oat roots [9]. Conceivably, because of the nature of the tissue, leaf vs. root, membrane ATPase activities might be expected to differ. On the other hand, microsomal preparations from corn roots were reported by Tipton et al. [25] to be comparable to the values presented in this report.

Several lines of evidence suggest that membrane ATPase may be involved as a

step in helminthosporoside-induced symptoms in susceptible sugarcane. These are: (i) membrane ATPase activity is reduced at least 50% after heat treatment (rendering normally susceptible leaves resistant) and this activity remains suppressed after actinomycin D or N_2 treatment (Table I); (ii) plots of symptom expression and membrane ATPase activity versus the reciprocal of the absolute temperature both show the same infection at 32°C (Fig. 1). The slopes of these two processes are not expected to be identical since the development of symptom expression represents a multitude of steps that each may be temperature dependent. (iii) Plots of symptom expression and membrane ATPase activity both slope downwardly as a function of the duration to exposure to 35°C (Fig. 2). Even though the slopes of the two processes are not parallel, they do show the same approximate sensitivity to exposure of caloric input at times of exposure beyond 1 h, indicating that the processes may be related. However, no apparent reduction in ATPase activity occurs before 1 h while there is a slight reduction in symptom production (Fig. 2). Again, this anomalous situation may be an expression of several heat-sensitive steps in the complex process of symptom expression. Overall, the data suggest that membrane ATPase is being heat denatured since the curve in Fig. 2 closely resembles the heat denaturation curve of membrane D-lactate oxidase found in *Escherichia coli* [26]; (iv) normally, helminthosporoside activates membrane ATPase in microsomal preparations [8] (Table II). The membranes of heat-treated leaves no longer show toxin activation of membrane ATPase; however, the activation phenomenon again occurs after the heat-treated leaves become susceptible (Table I). Thus by association of events, the two processes appear related; (v) general inhibitors and a specific inhibitor of membrane ATPase all cause complete protection of susceptible cane leaves, as well as a marked depression in the activity of membrane ATPase (Table III). Fluoride is known as an inhibitor of a number of enzymes including numerous phosphatases, oxidases, and kinases. On the other hand, octylguanidine is an inhibitor of Rb^+ uptake by roots according to Lepe and Avila [21]. These authors suggest that the mechanism of action of alkylguanidines is to interact with the membrane structure involved in the transport of Rb^+. Presumably, such a structure is the (K^+ + Mg^{2+})-ATPase associated with the plasma membrane of the plant cell since it is the enzyme most closely connected with regulated K^+ transport [9].

In the absence of K^+ and Mg^{2+}, the effects of helminthosporoside on susceptible cane protoplasts were greatly ameliorated. This would be expected of membrane (K^+ + Mg^{2+})-ATPase were having some critical role in toxin-mediated cellular death, since without these critical ions ATPase activity is greatly reduced [8,9]. These observations are also consistent with the in vivo protective effects rendered by EDTA (chelating agent) and its complete inhibition of ATPase activity (Table III).

Although not totally conclusive, the available evidence does support the involvement of membrane ATPase in toxin-mediated cellular death, but the mechanism of this involvement is unknown. The toxin does not activate ATPase in normally resistant clones and common α-galactosides structurally related to the toxin do not activate membrane ATPase [8]. We do know that the toxin does not interact directly with membrane ATPase [8]. Furthermore, there is probably not a binding protein-ATPase complex in the membranes

since Triton X-100 would not solubilize such a complex [27]. It is conceivable that the toxin-binding protein complex exerts its effects via the lipid bilayer ultimately altering the relationship of the membrane ATPase to its immediate lipid environment. The lipid environment around the ATPase does place some constraints on its activity as evidenced by a doubling of its activity in the presence of Triton X-100 [8]. The toxin activates membrane ATPase (Table II), perhaps to a greater extent in vivo than in vitro because of the adverse geometry and physiological state of the membrane vesicles in the test system. Nevertheless, the activation phenomenon may be a key event in the toxin-induced drop in membrane potential [29], toxin-induced changes in ion uptake [8], changes in permeability [6], and a 70% decrease in cellular ATP levels 30 min after toxin treatment (Pinkerton, F., unpublished observations). These are all suggestive of the possible involvement of the membrane ATPase in the process. Nevertheless, conclusive evidence will only be forthcoming when the membrane ATPase and binding protein are reconstituted into membrane vesicles and then examined for some of the effects observed above.

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