Inhibition of Plasma Membrane and Tonoplast H^+-Transporting ATPases by Glyceollin

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ABSTRACT

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The soybean phytoalexin glyceollin inhibits ATP-dependent proton transport associated with plasma membrane and tonoplast vesicles isolated from red beet (*Beta vulgaris* L.) storage tissue. Inhibition of proton transport by glyceollin in these vesicles was concentration dependent, and proton transport associated with tonoplast vesicles was more sensitive to the phytoalexin (50% inhibition at 50 μM) than that associated with plasma membrane vesicles (50% inhibition at 80 μM). When glyceollin was added to plasma membrane or tonoplast vesicles which developed a steady-state pH gradient, proton influx ceased; however, the gradient slowly dissipated. Glyceollin directly inhibited plasma membrane and tonoplast ATPase activity. The tonoplast ATPase was more sensitive to this compound than the plasma membrane ATPase. These results suggest that glyceollin could act to disrupt solute transport at both of these membranes through direct effects on the proton-pumping ATPases.

Additional keywords: *Glycine max*, membrane transport, mode of action.

Soybean plants (*Glycine max* L. Merr.) produce phytoalexins in response to a number of stimuli (5,15). Phytoalexins are low molecular weight, antimicrobial agents that are synthesized by the plant and accumulate after exposure to microorganisms (22). There is evidence that phytoalexins may play a major role in conferring disease resistance to the host plant (5 and references therein). Treatments that either physically (3) or chemically (20) reduce the amount of phytoalexin present in resistant plants cause a corresponding increase in sensitivity to the pathogen. Likewise, the transfer of phytoalexin along a wick from a genetically resistant variety to a genetically sensitive one enhances the resistance of the latter plant to the invading microorganism (3).

Glyceollin is a phytoalexin in soybeans that occurs in a series of at least three isomers (17). This compound may be responsible for the cultivar-specific resistance of soybeans to *Phytophthora megasperma* Drechsler f. sp. *glycinea* (13,28), and its accumulation has been correlated with the incompatible response of soybeans to *Meloidogyne incognita* (11) and *Pseudomonas syringae* pv. *glycinea* (14). This group of compounds has a broad range of toxicity to nematodes (12), fungi (15), insects (10), and bacteria (14). It has also been shown to inhibit electron transport in soybean mitochondria (1). Glyceollin (26) and other phytoalexins (4,8,18,24,27) also interact with cell membranes to disrupt membrane function and integrity. The general toxicity of this compound may serve to inhibit the growth of the invading pathogen by causing cell death in both the host (autophytotoxicity) and pathogen at the localized site of infection (1).

The plasma membrane and tonoplast constitute two major membrane barriers associated with the plant cells. The plasma membrane separates the exterior of the plant cell from the cytoplasm while the tonoplast surrounds the vacuolar components. Associated with both of these membranes are H^+ pumping ATPases, believed responsible for creating an electrochemical gradient across these membrane systems, which can then be used to drive secondary solute uptake (25). Although these plasma membrane and tonoplast ATPases have similar functions (H^+ pumping), their structure, inhibitor sensitivity, and enzyme mechanism differ substantially (6,25).

In this communication, the effect of glyceollin on proton transport in sealed plasma membrane and tonoplast vesicles isolated from red beet storage tissue (6,7) was examined. Red beet membranes were used because of their availability as a very well-defined system, and the lack of a suitable soybean vesicle membrane system. We are presently developing the soybean and *Phytophthora* systems to produce vesicles in order to determine whether a similar type of response occurs in these systems. The red beet vesicle system could be used to test effects of other phytoalexins, such as the sugarbeet phytoalexins betagarin and betavulgarin (19). Glyceollin inhibits proton transport in tonoplast and plasma membrane vesicles by a direct inhibition of their associated proton-translocating ATPases.

There is a general body of physiological data that indicates membrane leakage is caused by pterocarpan phytoalexins (4,8,18). Glyceollin effects on the tonoplast ATPase could lead to increased membrane permeability, dysfunction, leakage of contents of the vacuole, and death of the plant cell. Release of hydrolytic enzymes due to the breakdown of plasma membranes could affect fungal haustoria and intercellular and intracellular hyphae. This suggests that glyceollin-induced cell death may be involved in the interactions between soybeans and microorganisms, and involved in disease symptoms.

MATERIALS AND METHODS

Plant material. Red beet (*Beta vulgaris* L. 'Detroit Dark Red') storage roots were purchased commercially. The tops of the plants were removed and the storage roots were stored in moist vermiculite at 2–4 C until use. All root tissue used was stored at least 10 days to ensure uniformity in membrane isolation (23).

Preparation of glyceollin. Glyceollin, a mixture of isomers I, II, and III, was prepared using a modification of the methods of Boydston et al (1). It was purified by thin-layer chromatography followed by silica column chromatography. The quality of the glyceollin preparation was verified by high-pressure liquid chromatography and the absorbance spectrum (340–200 nm). The data presented is from preparations of glyceollin, which were predominantly isomer 1.

Membrane isolation. Sealed membrane vesicles were isolated according to a modification of the method of Giannini et al (7). Storage roots were peeled, cut into small squares, and then rapidly placed into a homogenization medium of 250 mM sucrose, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM sodium adenosine-5′-triphosphate (Na_2ATP), 1% (w/v) bovine serum albumin (BSA) (fraction V powder), 0.5% (w/v) polyvinylpyrrolidone (40,000 MW), 2 mM phenylmethylsulfonyl fluoride

(PMSF), 15 mM β-mercaptoethanol, 4 mM dithioerythretol (DTE), 10% glycerol, and 70 mM Tris/HCl, pH 8.0. DTE, PMSF, and β-mercaptoethanol were added to the medium just before use. The tissue was treated with homogenization medium before homogenization in a vegetable juice extractor by vacuum infiltration in a 1.5:1 (v:w) medium:tissue ratio for 5 min at ice temperature.

The homogenate was filtered through four layers of cheesecloth, centrifuged at 13,000 *g* (8,500 rpm) for 15 min in a Sorvall GSA rotor, and the pellet was discarded. The supernatant was centrifuged at 80,000 *g* (32,000 rpm) for 30 min in a Beckman type 35 rotor to obtain a microsomal membrane pellet. This pellet was suspended in a buffer consisting of 250 mM sucrose, 10% glycerol, 0.2% BSA, 1 mM DTE (added fresh), and 2 mM bistris propane (BTE), pH adjusted to 7.0, with solid 2[*N*-morpholino]ethanesulfonic acid (MES).

For the routine preparation of plasma membrane and tonoplast vesicles, the microsomal membrane suspension was layered on a discontinuous sucrose density gradient consisting of 15 ml of 26% (w/w) sucrose layered over 18 ml of 38% (w/w) sucrose. Both sucrose solutions contained 1 mM Tris/MES, pH 7.2, and 1 mM DTE. The gradient was centrifuged for 2 hr at 100,000 *g* (25,000 rpm) in a Beckman SW 28 rotor, and the membrane vesicles present at the 8.6/26% interface (tonoplast) and 26/38% interface (plasma membranes) were collected using a Pasteur pipet (7). Gradient-prepared membranes were diluted with an equal volume of suspension buffer and then centrifuged at 80,000 *g* for 30 min. The enriched membrane pellets were suspended in a suspension buffer at a protein concentration of approximately 2 mg/ml and either used immediately or frozen under liquid nitrogen and stored at −80 C. Membrane vesicles stored in this manner retained both transport and ATPase activity for up to 3 mo.

Enzyme assay. Adenosine triphosphatase activity was determined as described by Briskin and Poole (2). The assay for plasma membrane vesicles was carried out in a 1-ml solution of 3.75 mM ATP (Tris salt, pH 6.5), 3.75 mM $MgSO_4$, 1 mM NaN_3, 0.1 mM Na_2MoO_4, 100 mM KNO_3, 25 mM BTP/MES, pH 6.5, and 250 mM sorbitol. The assay for tonoplast ATPase activity was done in a similar manner, except that the KNO_3 was replaced by an equal concentration of KCl and the pH was 7.75. The assays were carried out at 38 C and 2 μM gramicidin D was present to prevent the production of a proton gradient and thus maximize the ATPase activity associated with the vesicles. After incubation for 20 min, the released inorganic phosphate was determined by the method of Ohnishi et al (21). Any further modifications are indicated in Results.

Optical measurement of proton transport. Proton transport in membrane vesicles was measured by the decrease in acridine orange dye absorbance (16). The assay conditions for proton transport in plasma membrane and tonoplast vesicles were identical to those used for the measurement of ATPase activity, except that 10 μM acridine orange was present and NaN_3 and Na_2MoO_4 were absent. The absorbance at 490 nm was measured at 22 C using a Beckman DU-40 spectrophotometer. Any variation in the reaction conditions are indicated in Results.

All data presented in this paper are representative of at least three repetitions with two different glyceollin preparations.

RESULTS AND DISCUSSION

The effect of glyceollin on proton transport in plasma membrane and tonoplast vesicles. In our previous studies (7), a technique was developed for the isolation of sealed plasma membrane and tonoplast vesicles from red beet storage tissue. In the presence of ATP and Mg^{2+}, these vesicles generate an acid-interior pH gradient (ΔpH) and positive-interior membrane potential ($\Delta\psi$) (6,7). The origin of this inwardly directed proton movement in these vesicles has been demonstrated to involve proton translocating ATPases associated with both of these membrane fractions (25). These ATPases and their associated proton pumping are believed to provide the driving force (proton electrochemical gradient or protonmotive force) necessary for energy-dependent solute movement across the plasma membrane and tonoplast.

Proton movement into vesicles can be measured using various optical techniques (16 and references therein). A common probe for measuring the formation of an acid interior pH gradient is acridine orange. This dye is a weak base that can move freely across the vesicle membrane in its unprotonated state. Upon the imposition of an acid-interior pH gradient, the dye accumulates in the interior of the vesicles and becomes concentrated. Under these conditions there is a reduction in absorbance at 490 nm. This reduction in *A* at 490 nm is proportional to the magnitude of the pH gradient across the vesicle membrane, so that it can be used as a measure of proton pumping for a vesicle population.

The effect of glyceollin on proton transport associated with tonoplast vesicles was examined (Fig. 1). After the establishment of an Mg:ATP-dependent pH gradient, the addition of up to 80 μM glyceollin did not result in a complete collapse of the gradient as was observed following the addition of gramicidin D, a channel-forming ionophore. Rather, glyceollin slowed the decrease in acridine orange absorbance, suggesting a more subtle effect on reducing the vesicle pH gradient than a complete disruption of membrane integrity. In the latter case, a rapid restoration of dye absorbance would be expected similar to that observed after the

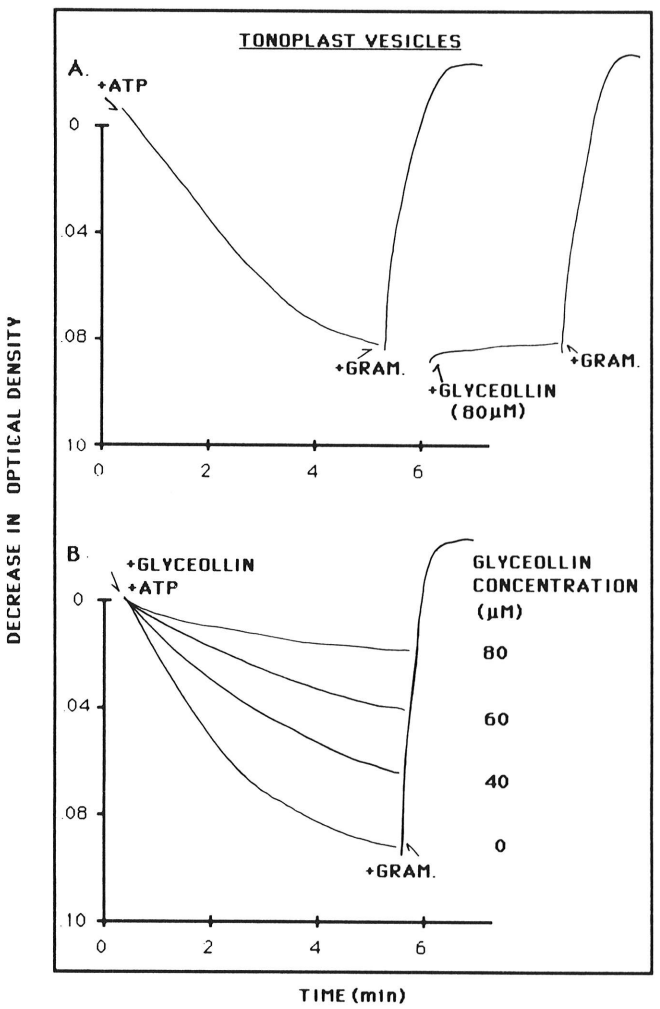

Fig. 1. Effect of glyceollin on proton transport in tonoplast vesicles from red beet storage tissue. Proton transport was measured by the 490 nm absorbance of acridine orange in an assay that contained 3.75 mM ATP (Tris salt, pH 6.5), 3.75 mM $MgSO_4$, 10 μM acridine orange, 250 mM sorbitol, 25 mM BTP/MES, pH 7.75, and membrane protein (25 μg of tonoplast protein or 100 μg of plasma membrane protein). The reaction was started by the addition of ATP and the pH gradient was collapsed by the addition of 5 μM gramicidin D. In trace A, glyceollin was added after the establishment of a pH gradient. In trace B, the indicated concentration of glyceollin was present in the assay initially. The change in absorbance caused by the addition of glyceollin was corrected in these assays.

addition of gramicidin D. When glyceollin was initially present in the proton transport assay, both the initial rate of proton transport and the final gradient (6 min) were reduced in a concentration-dependent manner. A 50% reduction in the initial rate of proton transport by glyceollin occurred at about 50 μM.

Glyceollin, at 80 μM, inhibited proton transport in plasma membrane vesicles without causing a total collapse of the pH gradient (Fig. 2). When glyceollin was added to plasma membrane vesicles at the start of the reaction, there was a concentration-dependent decrease in the initial rate of proton transport. The reduction in the rate of proton pumping for the plasma membrane vesicles due to glyceollin was not as large as that observed for the tonoplast vesicles (50% reduction in rate occurring at about 80 μM).

The effect of glyceollin on tonoplast and plasma membrane ATPase activity. While glyceollin can inhibit measured proton transport activity in plasma membrane and tonoplast vesicles, the nature of this effect is uncertain. The reduction of the pH gradient by glyceollin in these vesicles could occur either from a direct inhibitory effect on the ATPases associated with these membrane vesicles or through some change in proton conductance of the membrane itself. Because glyceollin could exert its effect on plasma membrane and tonoplast proton pumping in the initial phase of the assay where the back leakage of protons out of the vesicle would be of little significance, the latter effect is less likely. The ATPase activity associated with these membrane fractions was measured directly to test this inhibitory effect. The assay conditions for plasma membrane ATPase (pH 6.5, 100 mM KNO_3) and tonoplast ATPase (pH 7.75, 100 mM KCl) were optimized to eliminate interference from any cross-contamination of the respective ATPases and to provide a high level of enzyme activity. Inhibitors of mitochondrial ATPase (azide) and nonspecific phosphatase (molybdate) also were included in the assay to eliminate any contaminant activity of these components. Furthermore, the vesicles were made leaky to H^+ by the addition of gramicidin D so that no effects on proton gradients could feedback to modulate ATPase activity.

A progressive inhibition of phosphohydrolyase activity occurred when tonoplast ATPase activity was assayed in the presence of increasing concentrations of glyceollin (Fig. 3). A similar progressive inhibition was observed when plasma membrane ATPase activity was assayed in the presence of glyceollin except that the degree of inhibition was slightly less than that observed for tonoplast ATPase activity over the same concentration range. However, plasma membrane and tonoplast proton transport were affected by glyceollin in the same relative manner. These data suggest that the observed inhibition of proton transport by glyceollin in plasma membrane and tonoplast vesicles is primarily due to a direct inhibition of the tonoplast and plasma membrane H^+-ATPases by this group of compounds.

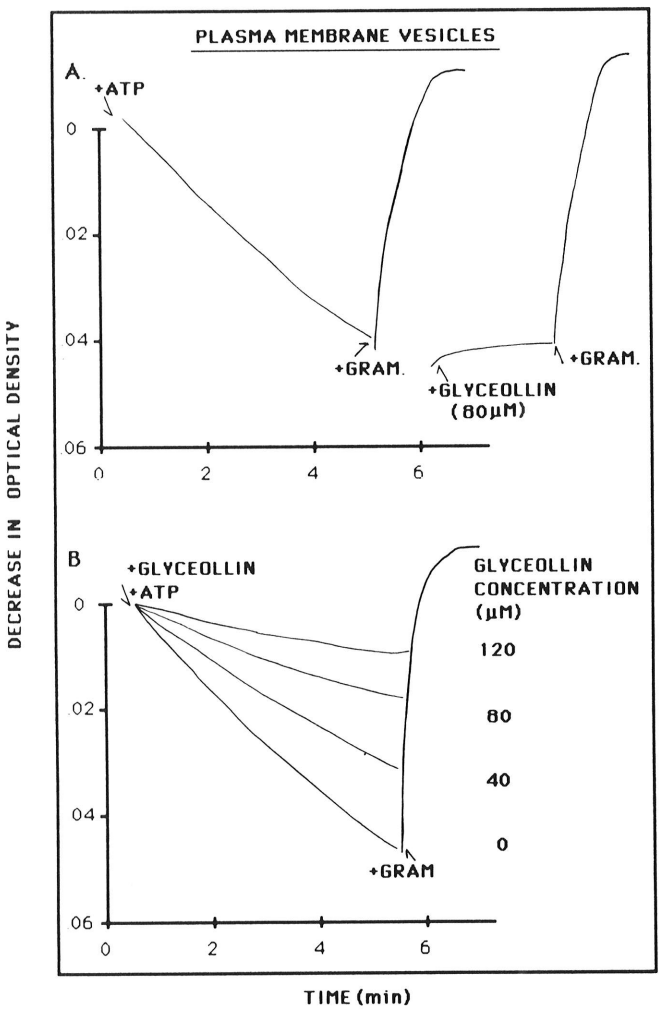

Fig. 2. Effect of glyceollin on proton transport in plasma membrane vesicles from red beet storage tissue. Proton transport was measured by the absorbance at 490 nm, of acridine orange. The transport assays were treated as described in Figure 1 with the plasma membrane vesicles, except that assay pH was 6.5.

CONCLUSION

The data presented demonstrate the ability of glyceollin, at observed physiological concentrations (1), to inhibit directly the plasma membrane and tonoplast ATPases of red beet storage tissue. Glyceollin does not directly disrupt membrane integrity since it did not cause the rapid collapse of a preformed vesicle pH gradient. This contrasts with reports from other laboratories that suggest a primary phytotoxic effect of other phytoalexins is the disruption of membrane integrity, which results in the leakage of cellular components (4,8,9,18). Previous studies by Hargraves (8) involving phaseollin effects on red beet storage tissue demonstrated that incubation with this phytoalexin caused electrolyte leakage and cellular death. When the tissue was examined by light microscopy, the first indication of cellular damage involved a disruption of the tonoplast in phaseollin-treated cells. Van Etten and Bateman (26) also described an increase in $^{86}Rb^+$ leakage from bean hypocotyl, preloaded with the radiotracer, following exposure to phaseollin. These authors concluded that the phytoalexin directly acted on plant membranes or some process required for membrane function. Glyceollin, a pterocarpan-type phytoalexin similar to phaseollin, causes the lysis of bovine

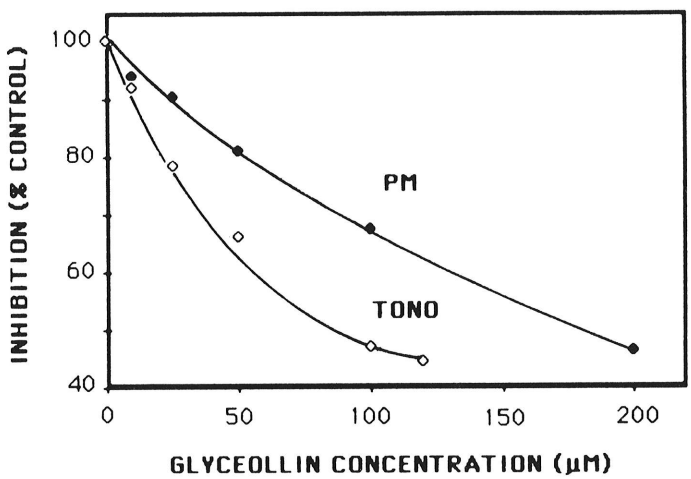

Fig. 3. Effect of glyceollin on plasma membrane and tonoplast ATPase activity. Plasma membrane and tonoplast fractions were assayed for ATPase activity in the presence of the indicated concentration of glyceollin. The standard assay contained 3.75 mM ATP (Tris salt, pH 6.5), 3.75 mM $MgSO_4$, 1 mM NaN_3, 0.1 mM Na_2MoO_4, 100 mM K^+ salt (KNO_3 for plasma membrane, KCl for tonoplast), 25 mM BTP/MES (pH 6.5 for plasma membrane; pH 7.75 for tonoplast), and 250 mM sorbitol, 40 μg of protein and was run at 38 C for 20 min.

erythrocytes (26) and hemoglobin leakage from rabbit erythrocytes (Paxton, *unpublished results*). The results are not consistent with glyceollin acting to directly disrupt membranes, but the results suggest a perturbation of basic bioenergetic processes essential for normal solute transport and compartmentation. This inhibitory effect on the energization of transport processes at the plasma membrane and tonoplast would certainly lead to leakage cell death.

Our experiments may suggest a general response of plant cells to pterocarpan phytoalexins. It will be of interest to see if fungal ATPases are similarly inhibited by these compounds, which would indicate a general mechanism for phytoalexin antibiotic activity. The results also demonstrate a differential sensitivity of plasma membrane and tonoplast ATPase to glyceollin in accordance with their differences in structure and enzyme mechanism.

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