Pergamon

0031-9422(95)00540-4

Phytochemistry, Vol. 40, No. 6, pp. 1655–1658, 1995

0031-9422/95 $9.50 + 0.00

THE EFFECT OF RISHITIN ON POTATO TONOPLAST VESICLE AND VACUOLE PROTON TRANSPORT

JOHN L. GIANNINI,*† MIKE NELSON* and GARY O. SPESSARD‡

*Department of Biology; ‡Department of Chemistry, St. Olaf College, Northfield, MN 55057-1098, U.S.A.

(*Received in revised form* 5 *April* 1995)

Key Word Index—Rishitin; potato; *Solanum tuberosum*; Solanaceae; proton leakage.

Abstract—Rishitin, a known potato phytoalexin, was tested for its effects on proton transport. Like the pterocarpan phytoalexins, glyceollin and phaseollin, rishitin was found to inhibit proton transport. At 100 μM rishitin, proton transport in potato tonoplast vesicles was inhibited by > 95%. This inhibition appears to be due to an increase in proton conductance and not to inhibition of the tonoplast ATPase. Potato vacuoles were also shown to have increased proton leakage in the presence of rishitin.

INTRODUCTION

Plants have various defense mechanisms for warding off pathogens. One common mechanism is the production of low-molecular-weight antimicrobial agents called phytoalexins [1]. There are over 100 phytoalexins that have been isolated with the majority being found in the dicotyledons [2]. Many have been shown to cause disruption of membrane function in both plants and fungi [3–5]. The importance of this disruption and its actual physiochemical cause is still a matter of debate.

The pterocarpan phytoalexins, glyceollin I (**1**), glyceollin III (**2**), and phaseollin (**3**), have been shown to cause inhibition of proton transport in isolated plant vacuoles and vesicles [6, 7]. This inhibition has been traced to the ability of these phytoalexins to cause a disruption in a membrane's ability to hold a proton gradient [6]. Studies have also suggested that phaseollin can allow larger molecular weight molecules to diffuse across vacuole membranes [6]. Because of the similarity in membrane action of the pterocarpan phytoalexins, it has been suggested that identifiable physiochemical properties responsible for membrane damage must exist. No clear understanding of these properties has yet been forthcoming.

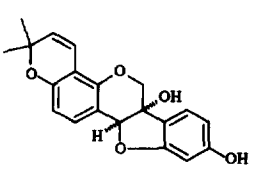
In the present study we were interested in determining if other phytoalexins, such as the terpenoids, could also cause inhibition of proton transport. Since terpenoid structure is, in general, quite different from that of the pterocarpans, a similar activity could help shed light on the physiochemical properties necessary for membrane damage. We decided to investigate the effect of the potato terpenoid, rishitin (**4**), for its effect on potato tonoplast proton transport.

†Author to whom correspondence should be addressed.

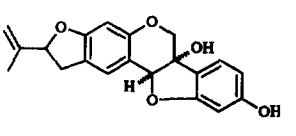
RESULTS AND DISCUSSION

In previous reports the effects of pterocarpan phytoalexins on membrane transport have been investigated. These studies showed that pterocarpans **1**, **2** and **3** caused a disruption in tonoplast proton transport [6]. The nature of this disruption was found to be an increase in the amount of H^+ conductance across the membrane. It was of interest to investigate whether terpenoid phytoalexins, such as rishitin, had a similar effect on the tonoplast of the plants in which they were produced.

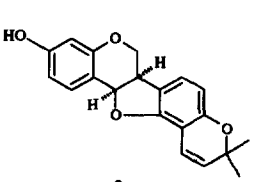
Potato tonoplast proton transport was inhibited by the presence of rishitin in the assay (Fig. 1). At a concentration of 75 μM rishitin, proton transport was inhibited by > 65%. When the concentration was raised to



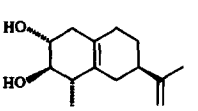
1



2



3



4

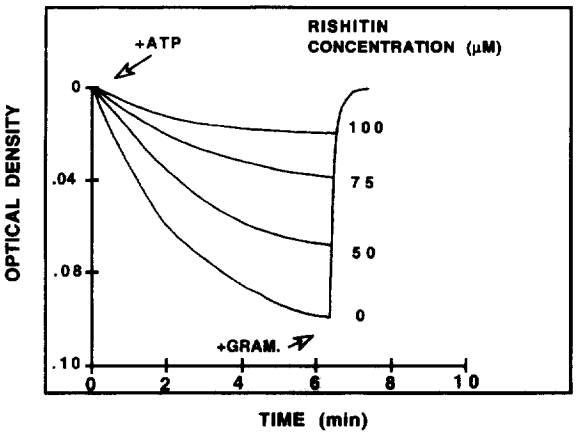


Fig. 1. Effect of rishitin on proton transport in potato tonoplast vesicles. Proton transport was assayed in the presence of increasing concentrations of rishitin. Assay conditions are described in the Experimental section using 32 μg of membrane protein. Proton gradients were collapsed by the addition of 3 μM gramicidin D(+ GRAM). Figures represent original spectrophotometric traces with readings taken every 5 sec.

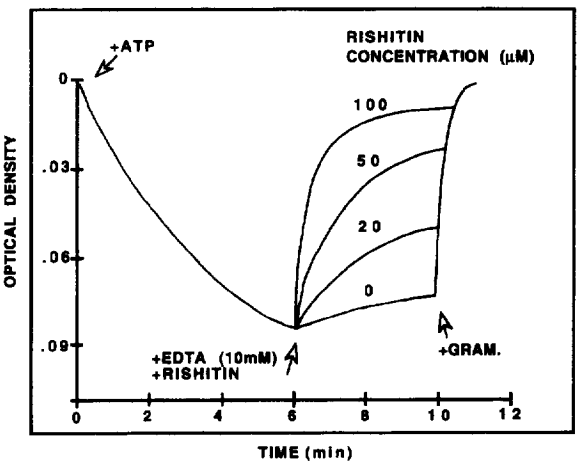


Fig. 2. Effect of rishitin on proton leakage in potato tonoplast vesicles. Proton leakage was assayed in the presence of increasing concentrations of rishitin added with 10 mM EDTA after 6 min of proton pumping. Proton transport was assayed using 32 μg of membrane protein. Proton gradients were collapsed by the addition of 3 μM gramicidin D(+ GRAM).

100 μM, proton transport was completely inhibited. These data suggested two possible reasons for rishitin's disruption of proton transport. The first would be an inhibition of the tonoplast proton pumping ATPase. The other possibility is that there was an increase in proton conductance due to rishitin at the tonoplast. Each of these possibilities was subsequently investigated.

Rishitin had little effect on the potato tonoplast ATPase (data not shown). At a concentration of 100 μM rishitin, only a small reduction (≈2%) in activity was observed. Clearly, rishitin was not inhibiting proton transport at the level of the ATPase.

The effect of rishitin on potato tonoplast proton conductance was also determined (Fig. 2). The presence of rishitin in the leakage assay caused an increase in proton conductance that was concentration-dependent. At 100 μM rishitin, proton conductance was so rapid that it resembled the effect observed if an ionophore (such as gramicidin D) were added. These data seem to indicate that rishitin affects proton transport primarily by increasing proton conductance.

As a final confirmation of our results, the effect of rishitin was tested on isolated potato vacuoles. These vacuoles were intact and quite active in proton transport. As was found with potato tonoplast vesicles, rishitin caused rapid increase in proton conductance when included in the assay (Fig. 3).

The effect of rishitin on proton transport of isolated potato vesicles and vacuoles is similar to that observed for the pterocarpan phytoalexins. The glyceollins (**1** and **2**) (I and III) and phaseollin (**3**) have both been shown to increase proton conductance of isolated vesicles and vacuoles from various plants [6, 7]. Phaseollin was also shown to cause larger molecules (β-anthocyanin) to leak from isolated red beet vesicles [6]. The observation that

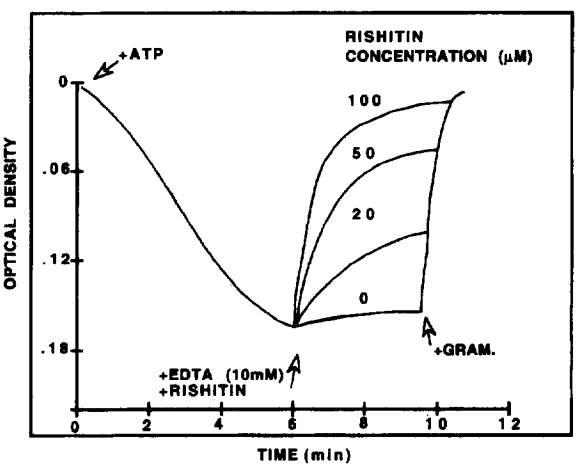


Fig. 3 Effect of rishitin on proton leakage in potato vacuoles. Assays were carried out using 100 μg of membrane protein. Proton gradients were collapsed by the addition of 3 μM gramicidin D(+ GRAM).

rishitin, a terpenoid phytoalexin with a very different structure than the pterocarpans, has a similar effect on proton leakage may suggest a common primary function for many phytoalexins in membrane disruption. The concentration of rishitin used in these studies is within the physiologically measured concentration of rishitin found in potato tubers infected with incompatible races of *Phytophthora infestans* [8]. Also, extremely high local concentrations (0.6 μmol ml^{-1}) of the phytoalexin glyceollin I have been measured in soybean roots near the site of infection [9]. This suggests that membrane disruption could be a primary effect of phytoalexin exposure.

Studies are underway to determine the physiochemical properties of pterocarpans that cause membrane perturbation. It will be interesting to see if these properties will also apply to other phytoalexin families such as the terpenoids.

In conclusion, it appears that rishitin (a terpenoid) inhibits proton transport by causing an increase in proton conductance. These results are similar to those observed for the pterocarpan phytoalexins.

EXPERIMENTAL

Plant material. Potatoes were purchased commercially and were stored at 4° for at least 24 hr prior to use in vesicle and vacuole isolations.

Tonoplast vesicle isolation. Potato tonoplast vesicles were isolated using a modification of the method of Ref. [6]. Tubers were peeled, cut into small squares, and then rapidly placed into a homogenization medium containing 250 mM sucrose, 70 mM Tris–HCl (pH 8.0), 10% glycerol, 0.5% polyvinyl pyrilidone (PVP-40 000 M_r), 3 mM EDTA, 2 mM phenylmethyl-suphonyl fluoride (PMSF) and 15 mM β-mercaptoethanol. PMSF and β-mercaptoethanol were added to the medium just prior to use. The homogenate was filtered through 4 layers of cheesecloth and then centrifuged at 13 000 *g* for 15 min. The 13 000 *g* pellet was discarded and the supernatant fraction was centrifuged at 80 000 *g* for 30 min. The pellet was suspended in 3 ml of the homogenization buffer and layered onto a 24% sucrose (w/w) layer. The preparations were centrifuged at 100 000 *g* for 2 hr. The interface between the homogenization buffer and the 24% sucrose layer was saved. This fraction was previously determined to be of tonoplast origin [10].

Vacuole isolation. Potato vacuoles were isolated using a modification of the method of Ref. [11]. Potatoes were peeled and cut into small squares, and then placed in an ice cold homogenization medium containing 1 M sorbitol, 5 mM EDTA, 0.5% (w/v) PVP, 50 mM Tris–Mes (pH 8.0) and 15 mM β-mercaptoethanol. The potatoes were then chopped with a food processor (Oscar, Sunbeam) for 30 sec. The homogenate was filtered through cheesecloth, then centrifuged at 20 *g* for 3 min at 4°. The pellet was discarded and the supernatant was centrifuged at 2500 *g* for 10 min at 4°. The supernatant was discarded and the pellets were suspended in 10 ml of 35% percol (v/v) in suspension medium. The suspension medium contained 1.2 M sorbitol, 25 mM Tris–Mes (pH 7.0), 1 mM EDTA. Suspension buffer (10 ml) was layered on top of the percol and the preparation was centrifuged at 19 000 *g* for 30 min at 4°. The percol/suspension buffer interface was collected and diluted × 2 in suspension buffer, then spun at 2500 *g* for 10 min. The resulting pellet containing vacuoles was suspended in 1 ml suspension buffer and kept on ice.

Protein assay. Protein was determined by the method of Ref. [12] using BSA as a standard.

Optical measurement of proton transport. Proton transport in membrane vesicles was measured by the decrease in acridine orange dye absorbance [6]. The assay was carried out in a 1 ml solution containing 250 mM sorbitol, 25 mM BTP–Mes (pH 7.0), 100 mM KCl, 3.75 mM $MgSO_4$, 3.75 mM ATP and 10 μM acridine orange. When proton transport was measured in vacuoles, 1.2 M sorbitol was used. The change in absorbance at 490 nm was measured at 22° with a Beckman DU-60 spectrophotometer.

Enzyme assay. Adenosine triphosphatase activity was determined as described previously [6]. The assay was carried out in a 1 ml solution containing 3.75 mM ATP, 3.75 mM $MgSO_4$, 100 mM KCl, 25 mM BTP–Mes (pH 7.0) and 250 mM sorbitol. Assays were carried out at 23° and 2.5 μM gramicidin D was added to prevent the production of a proton gradient and thus maximize the ATPase activity associated with the vesicles. Following incubation of the assay mixture for 20 min, the released inorganic phosphate was determined [13].

Isolation of rishitin (**4**). Potatoes (5–600 g) were washed with H_2O, soaked briefly in 70% EtOH in H_2O, and then cut into 0.5 cm thick slices. The slices were incubated at 15° for 24 hr. Next, a 20 mM solution of arachidonic acid was applied to the entire surface of each slice. Upon incubation for 96 hr at 15°, the slices were cut into smaller pieces and suspended under ethyl ether in a sealed chamber for 24 hr. Gravity filtration of the ethereal mixture produced a bilayered filtrate that was separated. The organic layer was concentrated under reduced pressure, and the resulting residue was suspended in 250 ml of Et_2O and 150 ml of H_2O. The organic layer was dried over $MgSO_4$ and concd under red. pres. to give *ca* 200 mg of crude rishitin extract. The extract was chromatographed over a 4 × 10 cm column of silica gel (60–200 mesh) using 600 ml of 1:1 hexane–EtOAc as the eluting solvent. Concentration of the eluate gave *ca* 60 mg of enriched rishitin residue that was then subjected to flash chromatography over 60 Å silica gel (2 × 8 cm column, 1:1 hexane–EtOAc eluting solvent, at least 15 × 10 ml fractions or 6 column vols were collected). The fractions were concentrated and the residue taken up in 5 ml of MeOH. The resulting solution was heated to boiling and treated with activated charcoal. Filtration of the mixture and concentration of the filtrate *in vacuo* gave 5–10 mg of rishitin: NMR $\delta(CDCl_3)$ 4.75 (broadened singlet, 1H, terminal vinylic), 4.62 (singlet, 1H, terminal vinylic), 3.57–3.72 (multiplet, 1H, J = 8 Hz, H–C–OH), 3.22 (triplet, 1H, J = 8–9 Hz, H–C–OH), 1.4–2.4 (complex multiplet, 10H, ring protons), 1.72 (singlet, 3H, =C–CH_3, and 1.14 ppm (doublet, 3H, 7 Hz, H–C–CH_3), IR (thin film) ν_{cm}^{-1} 3388 (*broad-strong*, *O*–H str), 1080 (medium, C–O str), 1040 (medium, C–O str), 890 (weak-medium, $=CH_2$). The IR and NMR spectra matched those of an authentic sample [14].

Proton leakage. Proton conductance was determined by first allowing the vesicles or vacuoles to pump protons for 6 min. At the end of this period, 10 mM EDTA was added to inhibit ATPase activity. Passive proton efflux was then monitored in the presence or absence of added rishitin.

Acknowledgements—The authors would like to thank Dave Tetzlaff for his assistance in the isolation of rishitin. The authors also acknowledge support from a National Institutes of Health–AREA grant and the Howard Hughes Medical Foundation.

REFERENCES

1. Deverall, B. J. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds), p. 1. Wiley, London.
2. Ersek, T. and Kiraly, Z. (1986) *Physiol. Plant* **68**, 343.
3. Hargreaves, J. A. (1980) *Physiol. Plant Pathol.* **16**, 351.
4. Giannini, J. L., Holt, J. S. and Briskin, D. P. (1990) *Plant Sci.* **68**, 39.
5. Van Etten, H. D. and Bateman, D. F. (1971) *Phytopathology* **61**, 1363.
6. Spessard, G. O., Hanson, C., Halvorson, J. S. and Giannini, J. L. (1994) *Phytochemistry* **35**, 43–47.
7. Giannini, J. L., Holt, J. S. and Briskin, D. P. (1991) *Plant Sci.* **74**, 203.
8. Sato, N., Kitazawa, K. and Tomiyama, K. (1971) *Physiol. Plant Pathol.* **1**, 289.
9. Gardner, H. W., Desjardins, A. E., McCormick, S. P. and Weisleder, D. (1994) *Phytochemistry* **37**, 1001.
10. Hellergren, J. and Widell, S. (1989) *Plant Physiol. Biochem.* **27**, 175.
11. Leigh, R. A. and Branton, D. (1976) *Plant Physiol.* **58**, 656.
12. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 243.
13. Ohnishi, T., Gali, R. S. and Mayer, M. L. (1979) *Anal. Biochem.* **69**, 261.
14. Hahn, M., Bonhoff, A. and Grisebach, H. (1985) *Plant Physiol.* **77**, 591.