

Differential Ion Stimulation of Plasmalemma Adenosine Triphosphatase from Leaf Epidermis and Mesophyll of *Nicotiana rustica* L.

Received for publication September 18, 1978 and in revised form January 2, 1979

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ABSTRACT

An ATPase preparation, presumed to be associated with plasma membrane due to the coincidence on isopycnic gradients of cellulase and β -glucan synthetase at high substrate, has been isolated from the epidermal and mesophyll of tobacco leaf. The ATPase from both tissues was found to prefer ATP over other nucleotides. The pH optimum was 7.0 in the presence of 3 millimolar $MgCl_2$ and pH 6.5 in the presence of 3 millimolar $MgCl_2$ and 100 millimolar KCl. Monovalent ion stimulation patterns of the ATPases from these tissues were found to differ and ion accumulation patterns in these tissues reflect this difference: mesophyll accumulated roughly equal amounts of Na^+ and Rb^+ and its plasma membrane ATPase is also equally stimulated by these ions; on the other hand, epidermal ATPase preparations showed a stronger stimulation by Rb^+ than Na^+ and this tissue was found to accumulate Rb^+ in preference to Na^+ . Abscisic acid and fusicoccin affected both ATPase activity and ion uptake, the former inhibiting and the latter stimulating these parameters. These data support the hypothesis that the epidermal plasmalemma ATPase is involved in stomatal opening.

The K^+ -stimulated Mg^{2+} ATPase associated with the plasma membrane is thought to have a role in ion transport into and compartmentalization within plant cells (2, 4, 7). A number of workers have characterized this enzyme from root and stem preparations but very few investigations have been carried out with photosynthetic tissue (1, 11) in spite of strong evidence suggesting its involvement with the regulation of stomatal opening (8, 19). Virtually all articles to date have dealt with homogenized organs (see the recent article by Leonard and Hotchkiss [16] for an exception). The plasma membrane ATPases from tissues within an organ could differ markedly (2), yet such differences would be made indistinguishable by homogenization. This may be especially true of leaf tissue, where the epidermis is thought to contribute monovalent ions to the guard cells for stomatal opening.

Plant leaves exhibit a differential accumulation of cations (3, 9, 19). Jacoby (10) found in bean leaves that Na^+ uptake in the light was concentrated primarily around the leaf veins, and that Rb^+ and K^+ absorption in the light was found exclusively in the stomatal complexes of the epidermis. This is in agreement with the known role of K^+ in stomatal opening (3, 9, 19). In many plants Na^+ may replace K^+ as an osmoticum leading to stomatal opening, but only at 10-fold higher concentrations. It is possible that control of the preferential uptake of one cation over another may be located in the cation-stimulated ATPases of the cell types

involved; if this is so, then plasma membrane ATPases from epidermal and mesophyll tissue would show different ion stimulation patterns. In the following paper we report just such a differential stimulation in an ATPase associated with a plasma membrane-enriched fraction prepared from the epidermis and mesophyll tissue of tobacco leaves.

MATERIALS AND METHODS

Plant Material. Tobacco plants were grown from seed in a phytotron with 16-h day, a day temperature of 23 C and night temperature of 17 C. The middle leaves of plants between the age of 2 and 3 months were used in the experiments. The lower epidermis was stripped and cleaned of adhering mesophyll cells by scraping with finger. The remaining leaf tissue, without the lower epidermis, was rolled and 1-mm-wide strips were cut with a razor blade (=leaf strips). For preparation of membrane fractions 1 g of the leaf strips and 70 cm² of the epidermis were used (Jacoby [10] has calculated that 71.5 cm² of leaf epidermis equals 1 g fresh weight). For ion uptake experiments 200 mg of leaf strips and 10 cm² of peeled epidermis were used.

Preparation of Membrane Fractions. Membrane fractions were obtained using the method of Hodges and Leonard (8). All procedures were carried out at 0 to 4 C. The epidermal strips and the leaf strips were homogenized separately with a mortar and pestle in a medium containing 0.25 M sucrose, 3 mM EDTA, 25 mM Tris-Mes (pH 7.2), and 3 mM dithioerythritol. The resulting brei was strained through four layers of cheesecloth and centrifuged at 13,000g for 15 min. The supernatant was next centrifuged for 30 min at 80,000g. The pellets so produced were resuspended in the homogenizing medium, combined, and again centrifuged at 80,000g for 30 min. This pellet was suspended in 18% (w/w) in 1 mM Tris-Mes (pH 7.2). One-half of the resuspension was layered on a 4.5-ml linear sucrose gradient, 20 to 50% (w/w) in 1 mM Tris-Mes (pH 7.2). This gradient was centrifuged 2 h in a Spinco SW 50.1 rotor at 100,000g. Fractions of 0.4 ml were collected using an ISCO model 184 gradient fractionator while simultaneously monitoring *A* at 660 nm and 280 nm.

Enzyme Determinations. All enzyme assays were carried out in a 0.5-ml reaction mixture. Enzyme assays utilizing phosphate substrates were carried out at 38 C in a solution containing 3 mM $MgSO_4$, 50 mM KCl, 3 mM phosphate substrate, and 33 mM Tris-Mes buffer at the appropriate pH. Before use, ATP was converted to the Tris form by Dowex 50-W ion exchange chromatography (8). Other nucleotides were purchased in the Tris form from Sigma. The Pi released was determined by the Fiske and Subbarow procedure (5).

NADH-Cyt *c* reductase was assayed at room temperature by

following the reduction of Cyt *c* at 550 nm (8). Latent IDPase activity in membrane fractions was determined by assaying for Pi released from Tris-IDP in 3 mM MgSO₄, 50 mM KCl, and 33 mM Tris-Mes at pH 7.5 (14). Membrane fractions were incubated for 4 days at 4 C prior to assay. Malic dehydrogenase was determined at pH 8.5 by following the oxidation of NADH at 338 nm (21). Glucan synthetase was assayed by the method of Van Der Woude *et al.* (20) as modified by Hendrix and Kennedy (6). Cellulase activity was assayed by the method of Koehler *et al.* (11). Protein was determined by the procedure of Lowry *et al.* (17).

Ion Absorption. Prior to the experiment the leaf strips and peeled epidermis were aerated for 1 h in 0.2 mM CaSO₄. All incubation media also contained 0.2 mM CaSO₄. Leaf strips or peeled epidermis were incubated 30 min in the light in 10 mM chlorides of ²²Na or ⁸⁶Rb at 30 C (0.1 μCi/μmol). After washing the tissue for 15 min in 0.2 mM CaSO₄, the radioactivity in the samples was determined by the Cerenkov radiation technique of Laüchli (12) after placing the tissue in 10 ml of 2.5 mM 7-amino-1,3-naphthalene disulfonic acid.

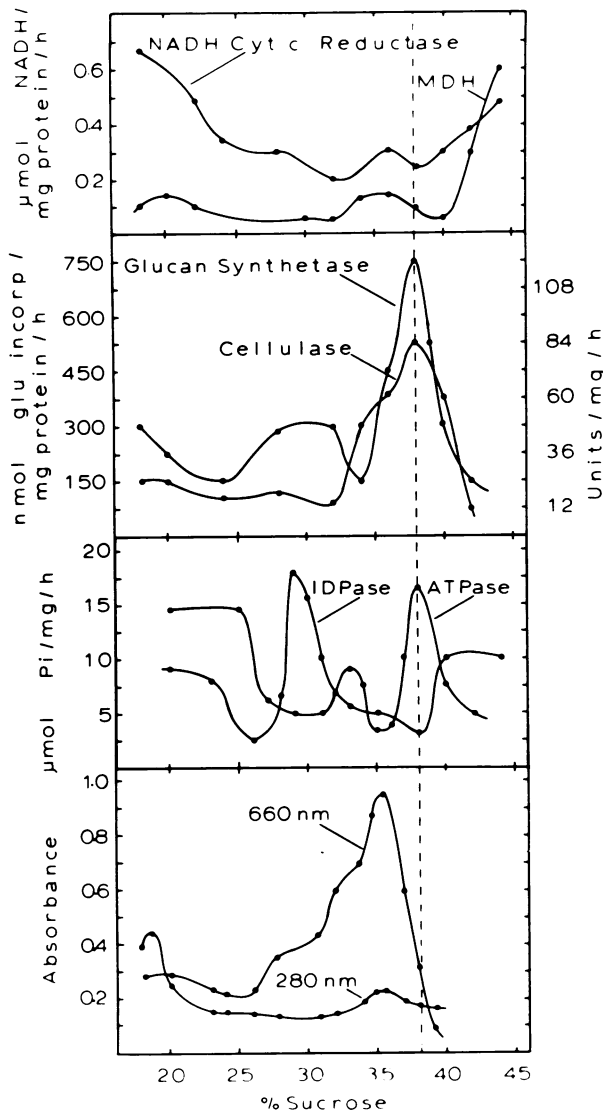


FIG. 1. Distribution of resuspended 13,000 to 80,000g K pellet from a mesophyll preparation on a linear isopycnic sucrose gradient (20–50%, w/w) after 2 h at 100,000g: *A*, at 280 nm (protein) and at 660 nm (Chl); ATPase activity at pH 6.5 in the presence of 3 mM MgSO₄ and 50 mM KCl; glucan synthetase activity as incorporation of [¹⁴C]glucose from 1 mM UDPG (plasma membrane); cellulase activity in viscosimetric units (plasma membrane); latent IDPase (Golgi membranes); malate dehydrogenase (mitochondria); NADH-Cyt *c* reductase (ER and mitochondrial).

ABA was purchased from Sigma. Fusicoccin was a gift from Dr. E. Marrè and Dr. A. Ballio.

RESULTS

The *A* and distribution of various enzymes associated with the 12–80,000g membrane fraction from mesophyll tissue along a linear 20 to 45% (w/w) sucrose density gradient are illustrated in Figure 1. There are three peaks of ATPase activity in this isopycnic sucrose gradient: at the top of the gradient, at 33% sucrose and at 38% sucrose (Fig. 1). Chloroplast fragments, as measured by Chl *A* at 660 nm, show a broad band from 31 to 37% sucrose, while Golgi membranes, as measured by latent IDPase activity, band at 29% sucrose (Fig. 1). Contamination of the ATPase peak at 38% sucrose by ER and mitochondrial fragments is minimal as shown by the low activity of NADH-Cyt *c* reductase and malic dehydrogenase at 38% sucrose (Fig. 1). The ATPase peak at 38% sucrose appears to be plasmalemma since glucan synthetase at high substrate levels and cellulase activity, both markers for plasmalemma (11, 20), also exhibit peak activity at 38% sucrose (Fig. 1). The membranes which banded at 38% sucrose were collected for further study. The same distribution of membrane-bound ATPases and enzyme markers was found in epidermal fractions (data not shown).

Figure 2 shows the pH optimum of the plasmalemma ATPase from a mesophyll preparation. The Mg-activated ATPase has its highest activity at pH 7; above pH 7 the activity decreases. With an addition of 100 mM KCl, the K⁺-enhanced ATPase activity is highest at pH 6.5 with some enhancement also present at pH 7; above pH 7 ATPase enhancement due to K⁺ is negligible.

Both the mesophyll and epidermal preparation were examined for specificity toward ATP with similar results (data shown for mesophyll preparation). As seen in Table I, ATP gave the highest phosphatase activity at 27 μmol Pi/mg protein · h. The next highest activity was found with ADP which was 18% that of ATP. All other nucleotides tested showed activity lower than with ADP.

The two membrane preparations were examined for specificity toward K⁺ as the stimulating ion (Table II). In mesophyll preparations K⁺ showed the highest stimulation, with Rb⁺ and Na⁺ both showing 90% of the K⁺ stimulation. Lithium and Tris chloride exhibited much less stimulation of the ATPase, approximately 50% of that given by K⁺.

The ion stimulation pattern is different in the epidermal fraction. The chlorides of lithium and Tris still exhibit around 50% of the stimulatory activity caused by K⁺; however, Na⁺ exhibits only 70% of the K⁺ activity while Rb⁺ exhibits 112% of the K⁺ activity.

These differences between the sensitivity of the ATPases of mesophyll or epidermal plasmalemma to Na⁺ and Rb⁺ are also seen in the ion uptake characteristics of the two tissues. The

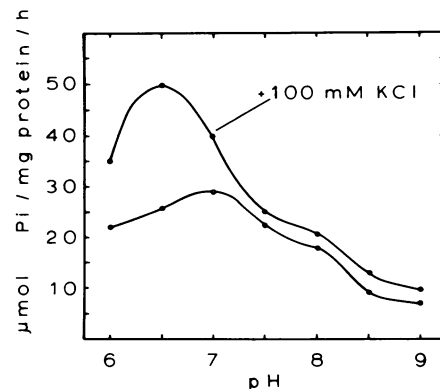


FIG. 2. pH optimum of ATPase associated with membranes from a mesophyll preparation which band at 38% sucrose on isopycnic gradients. (○): ATPase activity in presence of 3 mM MgCl₂; (●): activity in presence of 3 mM MgCl₂ and 100 mM KCl.

Table I. Nucleotide specificity of the membrane-bound ATPase activity from tobacco mesophyll which migrates to 38% sucrose in isopycnic gradients. The liberation of Pi from the tris salt of various phosphate substrates at 3 mM was determined at 38 C and pH 6.5. Each reaction mixture contained, in addition, 3 mM MgSO₄ and 33 mM tris-MES. Specific activity is presented in μ moles Pi released/mg protein/h (\pm SD). Numbers in parentheses are activities relative to ATP. Data represent means of six replicates, \pm SD.

Substrate	Specific ATPase Activity
ATP	27 \pm 2.0 (100)
ADP	5 \pm 0.3 (18)
GTP	3 \pm 0.1 (11)
UTP	2 \pm 0.2 (7)
CTP	1 \pm 0.1 (3)

Table II. Monovalent ion stimulation of ATPase activity from epidermal and mesophyll preparations.

Relative ATPase activity of membranes taken from the 38% (w/w) location on isopycnic gradients was measured in the presence of 3 mM MgSO₄, 3 mM tris-ATP and 33 mM tris-MES, pH 6.5, plus 50 mM of the respective chloride salt.

Chloride Salt	ATPase activity as per cent of KCl stimulation	
	Mesophyll	Epidermis
KCl	100	100
RbCl	90	112
NaCl	90	70
LiCl	54	45
Tris Cl	45	51

Table III. The uptake of ²²NaCl and ⁸⁶RbCl by isolated leaf epidermis of mesophyll in light.

Results are expressed as μ moles/g fresh wt/hr. Data represent means of five replicates, \pm SD.

Tissue	Na	Rb
Mesophyll	4.1 \pm 0.03	3.4 \pm 0.14
Epidermis	0.1 \pm 0.03	0.6 \pm 0.02

Table IV. The effect of abscisic acid and fusicocin on the membrane-bound ATPase activity and upon Rb uptake in epidermis and mesophyll preparations for tobacco.

The concentration of abscisic acid and fusicocin was 10 μ molar. Numbers in parentheses represent relative activities. Data represent means of five replicates \pm SD.

Treatment	Rb uptake, μ mole/g fresh wt/h		ATPase activity μ mole Pi/mg protein/h	
	Mesophyll	Epidermis	Mesophyll	Epidermis
No addition	3.2 \pm 0.1 (100)	0.8 \pm 0.02 (100)	22 \pm 2 (100)	26 \pm 2 (100)
Abscisic acid	2.4 \pm 0.2 (75)	0.2 \pm 0.01 (25)	18 \pm 1.5 (81)	10 \pm 1 (38)
Fusicocin	6.1 \pm 0.4 (190)	1.3 \pm 0.02 (162)	37 \pm 2 (168)	41 \pm 3 (157)

uptake of labeled Na^+ or RbCl into mesophyll cells is similar although the Rb^+ uptake is only 82% that of Na^+ (Table III); however, in isolated epidermis the uptake of Rb^+ is six times higher than that of Na^+ .

The total uptake in the epidermal tissue is much lower than that of mesophyll tissue (Table III). An explanation for this might be that in the epidermis the primary cells active in ion uptake are the guard cells (10) which constitute about 10% of the total epidermis.

Rb^+ uptake and Rb^+ stimulation of the plasmalemma ATPase in mesophyll and epidermis were also examined in the presence of ABA and fusicoccin. ABA induced stomatal closure and is thought to act at the level of ion uptake into the guard cells (19), while fusicoccin is known to enhance a H^+/K^+ exchange in plant tissue (1). Table IV shows that these compounds affected the plasma membrane ATPase from both mesophyll and epidermis although the effect of ABA was consistently greater in epidermis preparations. ABA at $10 \mu\text{M}$ inhibited Rb^+ uptake into the mesophyll by 25% and uptake into the epidermis by 75%. The mesophyll ATPase was inhibited 19% by ABA at $10 \mu\text{M}$ while the epidermal ATPase was inhibited 62%.

Fusicoccin stimulated ion uptake and ATPase in both tissues. Rb^+ uptake into the mesophyll was stimulated 90% and uptake into the epidermis was 62% higher than in controls. Mesophyll plasma membrane ATPase showed a 68% and mesophyll preparations a 57% stimulation in the presence of fusicoccin.

DISCUSSION

The isopycnic gradients from epidermis and mesophyll tissue both exhibited a plasmalemma ATPase with peak activity at 38% sucrose. The position of the peak ATPase activity agrees well with plasma membrane isolated from other plant tissues (8, 11, 16, 20) and the enzyme markers cellulase and glucan synthetase support its identity as plasmalemma. This fraction is also apparently relatively free of contaminating membranes, particularly chloroplast fragments which comprise a lighter fraction immediately above the plasmalemma.

The pH optimum of this membrane-bound ATPase is pH 7 in the presence of Mg^{2+} alone and pH 6.5 in the presence of Mg^{2+} and KCl , similar to the plasmalemma ATPase from root cells (6, 8, 15). This is in contrast to data obtained by Raghavendra *et al.* (18), where epidermal ATPase showed two optima at pH 5.5 and 7.5 in the presence of Mg^{2+} and Mg^{2+} plus K^+ , respectively. The latter work involved a relatively crude extract from epidermal strips of *Commelina beghalensis*, which may account for the difference between their results and those cited above. It is not clear that the ATPase that was measured in the *Commelina* was indeed associated with a plasmalemma-enriched fraction.

The ATPase preparations from tobacco epidermis and mesophyll showed different stimulation by monovalent cations. In particular, the epidermal ATPase was less stimulated by Na^+ than the mesophyll preparation. This was also reflected in the ion uptake studies of the two tissues; mesophyll tissue showed a slight preference for Na^+ over Rb^+ in uptake studies but in the epidermal preparations, Rb^+ uptake was six times as great as Na^+ uptake (Table III).

One might expect a 1:1 relationship between tissue ion uptake and ion stimulation of the plasma membrane ATPase if transmembrane ion transport is a function of ion-stimulated ATPase activity (4, 13). Such a correlation is found in mesophyll preparations (Tables II and III) but less quantitative agreement is found in the data from epidermal preparations (Tables II and III). However, if Na^+ but not K^+ (Rb^+) is extruded from tobacco epidermal cells, as has been reported for other plant tissues (7), one would not expect agreement between the ratio of ATPase stimulation by Na^+

and Rb^+ and the ratio of tissue accumulation by the same ions.

From the epidermal ion uptake data, it appears that the cells in the epidermis actively engaged in ion absorption are the guard cells. It is known that part of the stomatal opening process involves K^+ (or Rb^+) uptake and that this ion is more efficient than Na^+ in causing stomatal opening. If the epidermal ATPase is concerned with K^+ influx into the guard cells, then compounds affecting stomatal opening might also affect the ATPase activity. This is shown to be true in the case of fusicoccin and ABA. Fusicoccin, known to stimulate stomatal opening, also stimulates ATPase preparations and ion uptake in both mesophyll and epidermis. Beffagna *et al.* (1) found a similar stimulatory effect on K^+ -activated ATPases in crude extracts from spinach leaves. ABA, which inhibits stomatal opening, likewise inhibits epidermal ATPase and ion uptake. Ion uptake by mesophyll cells and ATPase isolated from mesophyll cells are also inhibited but to a lesser extent.

Plasmalemma ATPases seem to have a role in transporting ions into the mesophyll and epidermal cells of leaves. The differing behavior of these ATPases toward monovalent cation stimulation is reflected in the ion absorption patterns of the different tissues. Compounds which inhibit or stimulate K^+ or Rb^+ uptake and are known to alter stomatal opening also inhibit or stimulate the ATPases. One of these compounds, ABA, has a far greater effect on epidermal than on mesophyll preparations. We speculate that the epidermal ATPase we have isolated plays a role in the ion uptake leading to stomatal opening in tobacco leaves.

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