Modulation of H⁺-ATPase Activity by Fusicoccin in Plasma Membrane Vesicles from Oat (Avena sativa L.) Roots

A Comparison of Modulation by Fusicoccin, Trypsin, and Lysophosphatidylcholine

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The fungal phytotoxin fusicoccin affects various transport processes in the plasma membrane of plant cells. The plasma membrane (PM) H⁺-ATPase (EC 3.6.1.35) seems to be the primary target of fusicoccin action. The kinetics of the stimulation of the PM H⁺-ATPase by fusicoccin was studied in PM vesicles isolated from oat (Avena sativa cv Adamo) roots by aqueous two-phase partitioning.

Fusicoccin treatment shifted the pH optimum of the ATPase toward more alkaline values and increased V_max. No effects on K_m were observed. Treatment with trypsin resulted in stimulation of ATPase activity in control vesicles but not in the fusicoccin-treated vesicles. The characteristics of stimulation by trypsin in control vesicles were comparable with those of stimulation by fusicoccin. This result and the change of the polypeptide pattern on western blots suggest the involvement of the C-terminal inhibitory domain in the fusicoccin signal transduction chain. On the other hand, stimulation by lyso-PC demonstrated other characteristics than stimulation by fusicoccin. Lyso-PC was able to stimulate ATPase activity at both acidic and alkaline pH values. Kinetic analysis of the pH dependency curves revealed different mechanisms for activation by fusicoccin and by lyso-PC. Whereas fusicoccin shifted the pH dependency of formation of phosphorylated intermediate to more alkaline values, lyso-PC seemed to increase dephosphorylation independently of pH.

The PM H⁺-ATPase is a key enzyme in the response of the plant cell to various external and internal stimuli. It plays an important role in cell expansion, initiation of cell division, and responses of the cell to light, phytohormones, nutritional status, and phytotoxins (Serrano, 1989).

Recently, important advances have been made in unraveling the molecular mechanisms of the regulation of this enzyme. Like other ion-translocating P-type ATPases, the C terminus of the polypeptide seems to be important in the regulation of its activity (Palmgren et al., 1990). Activation of the PM H⁺-ATPase by lyso-PC is mediated by the interaction of the C terminus of the polypeptide with the lyso-phospholipid

(Palmgren et al., 1988, 1990; Palmgren and Sommarin, 1989). The C terminus of the native PM H⁺-ATPase contains an autoinhibitory domain (Palmgren et al., 1991), which puts a constraint on the activity of the enzyme. The conformational change, provoked by lyso-PC, results in the release of this constraint. Removal of the domain by a mild trypsin treatment also results in the activation of the enzyme (Palmgren et al., 1990). The inhibitory domain could be part of a regulatory domain that serves as the ultimate target for various stimuli (Palmgren et al., 1991). In yeast, the C terminus is also involved in the activation of the PM H⁺-ATPase by Glc (Serrano, 1983). Mutations in a potential phosphorylation site, present in the C terminus, result in reduced enzyme activation by Glc (Portillo et al., 1991).

The phytotoxin FC causes auxin-like responses in plant tissues (Marré, 1979). It promotes growth and affects ion fluxes across the PM. Although alternative explanations have been proposed (Blatt and Clint, 1989), these responses are generally ascribed to stimulation of the PM H⁺-ATPase (Marré, 1979). Several studies have demonstrated the effects of FC on ATP hydrolysis (Befagna et al., 1977; Blum et al., 1988; Schulz et al., 1990; De Michielis et al., 1991) and proton-translocating activity of the enzyme (Rasi-Caldogno and Pugliarello, 1985; Rasi-Caldogno et al., 1986; Aducci et al., 1988; Marra et al., 1992). In patch-clamp experiments FC induces positive outward currents (Serrano et al., 1988; Assmann and Schwartz, 1992; Lohse and Hedrich, 1992). These currents are sensitive to ATP depletion, dicyclohexylcarbodiimide, vanadate, and carbononylcyanide m-chlorophenylhydrazone, indicating the PM H⁺-ATPase as the current source (Serrano et al., 1988; Lohse and Hedrich, 1992).

The nature of the signal transduction chain between the FCBP and the PM H⁺-ATPase and its relation to the signal transduction chain of auxins are still largely unknown. It has been demonstrated that ATPase and the FCBP apparently are different entities (De Boer et al., 1989; Schulz et al., 1990). Also, the FCBP differs from the auxin-binding protein (Barbier-Brygoo et al., 1989).

The present study was performed to elucidate the molecu-
ular mechanism of the modulation of PM H\(^+\)-ATPase activity by FC and to analyze the effects of FC, trypsin, and lyso-PC on the PM H\(^+\)-ATPase in *Avena sativa* roots. It is shown that PM vesicles from FC-treated *Avena* roots have a shifted pH profile for ATPase activity that matches those induced by treatment of the control vesicles with trypsin. The similarity between the effects of in vivo FC treatment and in vitro trypsin treatment have been noted in two papers published recently, in which different materials were used (Johansson et al., 1993; Rasi-Caldogno et al., 1993). In addition, differences in the effects of treatment with lyso-PC, FC, and trypsin are demonstrated. Finally, the step in the ATPase activity that might be altered by FC and trypsin or by lyso-PC is identified by a kinetics analysis.

**MATERIALS AND METHODS**

**Plant Material**

Oat seeds (*Avena sativa* L. cv Adamo) were rinsed with running tap water for approximately 2 h at 30°C. The grains were sown on a stainless steel grid, placed above 10 L of tap water, and covered with wet filter paper. The germinating seeds were placed in a growth chamber in continuous darkness at a temperature of approximately 21°C. After 2 d the filter paper was removed, and the solution was substituted with 1 mM CaSO\(_4\) (minimum medium). This medium was continuously aerated and refreshed every 2 d.

**Isolation of the PM Vesicles**

Roots were harvested after 8 d. After the roots were cut from the lower side of the grid, they were stored in a container filled with minimum medium. Roots were carefully blotted dry, and fresh weight was determined. Then, the roots were transferred to aerated minimum medium with or without 10\(^{-5}\) M FC and incubated for 30 min at room temperature. PM vesicles isolated from root tissues that were treated with FC will be referred to as FC-treated vesicles, or PM + FC, and those isolated from roots not treated with FC will be referred to as control vesicles or PM - FC. PM vesicles were isolated by aqueous two-phase partitioning at 4°C according to the method of Larsson et al. (1987) as modified by De Boer et al. (1989).

Briefly, 200 g of roots were cut into 1- to 2-cm-long sections and transferred to 200 mL of homogenization medium (25 mM Mops, 5 mM EDTA, 250 mM Suc, 2 mM DTT, 10 mM KF, 3 mM Na\(_3\)ATP, 1 mM PMSF, 2 g L\(^{-1}\) casein, 2 g L\(^{-1}\) BSA, adjusted to pH 7.5 with BTP). The roots were homogenized in a Braun homogenizer equipped with razor blades. The homogenate was filtered through two layers of Miracloth. The residue was extracted again with 200 mL of homogenization medium in the Braun homogenizer. Pooled filtrates were centrifuged (20 min at 10,000g, and the resulting supernatant was centrifuged again (60 min at 50,000g). The pellet was resuspended in storage buffer and centrifuged again. The final pellet was resuspended in storage buffer, supplemented with 1 \(\mu\)g mL\(^{-1}\) of pepstatin and 1 \(\mu\)g mL\(^{-1}\) of leupeptin, to a final protein concentration of 4 to 6 \(\mu\)g mL\(^{-1}\). After freezing under liquid nitrogen, PMs were stored at \(-80°C\) until used for further experimentation.

**Enzyme Assays**

Protein content was determined with the Bio-Rad microassay using BSA as a standard. ATPase activity was measured as the release of Pi from ATP, after an incubation of 30 min at 30°C, according to the method of Staal et al. (1991). Some modifications were introduced. The incubation was stopped by the addition of 0.375 M H\(_2\)SO\(_4\), 0.75% (w/v) (NH\(_4\)\(_2\))Mo\(_7\)O\(_{24}\), 3% (w/v) FeSO\(_4\), and 0.75% (w/v) SDS, instead of using 33% (w/v) TCA. Brij-58 (0.0125% [w/v]) was used as a detergent. Mops-BTP (50 mM, pH \(\geq 7.0\)) and Mes-BTP (50 mM, pH < 7.0) were used as pH buffers. Latent IDPase was determined according to the method of Ray (1979). The release of Pi from IDP was determined according to the procedure of Coccuci and Marrè (1984). Glucan II synthase was determined according to the method of Ray (1979), as modified by Kaus and Jeblick (1985). Cyt c oxidase was determined according to the method of Appelmans et al. (1955). Anti-myacin A-resistant NADH-Cyt c reductase was determined according to the method of Hodges and Leonard (1974).

**Trypsin Treatment**

PMs were diluted in assay buffer of 25 mM Mops, 250 mM Suc, 5 mM EDTA, 2 mM ATP, and 0.04% (w/v) Brij-58 to a final protein concentration of 1 \(\mu\)g mL\(^{-1}\). This solution was mixed with an equal volume of assay buffer containing 0.04 \(\mu\)g mL\(^{-1}\) of trypsin. PMs were incubated for 45 min at 20°C. Proteolysis was stopped by adding one-tenth of the incubation volume of assay buffer with 4 \(\mu\)L of trypsin inhibitor. In controls trypsin and trypsin inhibitor were omitted, or trypsin inhibitor was added before addition of the PMs.

**SDS-PAGE**

PM protein (2 \(\mu\)g) was dissolved in 30 \(\mu\)L of sample buffer (10% [v/v] glycerol, 5% [v/v] mercaptoethanol, 2% [w/v] SDS, 10 mM EGTA, 0.5 mM PMSF, 0.02% [w/v] bromphenol blue, 62.5 mM Tris-HCl, pH 6.8) at 20°C for 15 min. Electrophoresis was performed according to the method of Laemmli (1970) on a Mini-Protean II (Bio-Rad). The discontinuous gel system consisted of a 4% stacking gel and 7.5% resolving gel. Cross-linkage was 2.7% in both gels.

**Analysis of the Polypeptides by Western Blotting**

After gel electrophoresis, the polypeptides were electro- phoretically transferred to an Immobilon PVDF membrane (Millipore) using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. Transfer buffer contained 25 mM Tris, 192 mM Gly. Transfer was performed at a constant 100 V for 1 h at 4°C. The PVDF membrane was incubated for 1 h at room temperature with 5% nonfat milk in TBS-T before incubation with the primary antibody. After three washes with TBS-T, the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase. The blots were visualized using chemiluminescence reagents.
temperature in blocking buffer (3% [w/v] BSA and 0.1% [v/v] Triton X-100 in TBS: 9% [w/v] NaCl, 10 mM Tris-HCl, 0.02% [w/v] NaN3 [pH 7.2]). Thereafter, the PVDF membrane was incubated overnight with a rabbit antiserum (No. 721, 0.02% [w/v] NaN3 [pH 7.2]). The antiserum was a gift from Dr. R. Serrano (Universidad Politécnica, Valencia, Spain). Afterward, the PVDF membrane was washed four times with 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100 in TBS and incubated for 1 h with the secondary antibody (goat anti-rabbit conjugated with alkaline phosphatase) in TBS, 0.1% (v/v) Triton X-100, and 1% (w/v) BSA. After the reaction buffer (0.1 M MgCl2, 0.1 M NaCl, 50 mM MgCl2 [pH 9.5]), it was incubated in reaction buffer with 0.165 mg mL⁻¹ of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and 0.33 mg mL⁻¹ of nitroblue tetrazolium chloride at room temperature. The bands on the membrane were quantified with a LKB Ultrascan XL (Bromma, Sweden).

Fitting of the Concentration and pH Dependency
The concentration and pH dependency of the ATP hydrolysis was evaluated by fitting the appropriate equations using the Marquardt nonlinear algorithm for least squares estimation of nonlinear parameters (Marquardt, 1963) with explicit weighting.

Statistics
All data presented are the means (±sD) of three independent PM isolations with two independent experiments for each isolation or otherwise as stated.

RESULTS
The Purity of the PM Fraction
To characterize the vesicles isolated by aqueous two-phase partitioning, the effects of inhibitors of various ATP-hydrolyzing enzymes in the microsomal fraction and the PM fraction were studied (Table I). The following inhibitors were used: azide for mitochondrial ATPase activity, bafilomycin and nitrate for vacuolar ATPase activity, molybdate for unspecific acid phosphatase activity, and vanadate for PM ATPase activity. At pH 7.5 the inhibitors of vacuolar ATPase activity, bafilomycin and nitrate, inhibited ATPase activity in the microsomal fraction by 6 and 30%, respectively. No inhibition by those two inhibitors was observed in the PM fraction. At pH 6.5 molybdate, azide, and vanadate inhibited microsomal ATPase activity by 6, 30, and 61%, respectively. In the PM fraction ATPase activity was inhibited only by vanadate (83%). Vanadate-sensitive ATPase activity showed a 2.7-fold enrichment after two-phase partitioning (Table II). The behavior of other marker enzymes during two-phase partitioning is shown in Table II. Enrichment of the PM marker glucan II synthase was 2.5-fold, which is similar to the enrichment of vanadate-sensitive ATPase activity. Both Cyt c oxidase and NADH-dependent Cyt c reductase showed a considerable reduction in activity. No reduction was ob-

### Table I. Effects of inhibitors and absence of ligands on ATPase activity in microsomes and PMs

Membranes were isolated from Avena roots. ATPase activity was determined at an ATP concentration of 5 × 10⁻⁹ M. Activities are the means (±sD) of at least four isolates, with two independent experiments for each isolate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Microsome ATPase Activity</th>
<th>PM ATPase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.84 ± 0.01</td>
<td>3.62 ± 0.08</td>
</tr>
<tr>
<td>-MgSO₄</td>
<td>0.04 ± 0.03</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>-K₂SO₄</td>
<td>1.65 ± 0.01</td>
<td>3.25 ± 0.04</td>
</tr>
<tr>
<td>-MgSO₄ - K₂SO₄</td>
<td>0.07 ± 0.00</td>
<td>-0.01 ± 0.01</td>
</tr>
<tr>
<td>+Na₂VO₅ (0.5 mM)</td>
<td>0.71 ± 0.04</td>
<td>0.61 ± 0.07</td>
</tr>
<tr>
<td>+Na₂MoO₄ (0.1 mM)</td>
<td>1.74 ± 0.05</td>
<td>3.62 ± 0.07</td>
</tr>
<tr>
<td>+NaN₃ (1 mM)</td>
<td>1.29 ± 0.02</td>
<td>3.77 ± 0.04</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.27 ± 0.22</td>
<td>1.35 ± 0.33</td>
</tr>
<tr>
<td>+Bafilomycin (0.1 μM)</td>
<td>1.20 ± 0.20</td>
<td>1.36 ± 0.35</td>
</tr>
<tr>
<td>+NaNO₃ (50 mM)</td>
<td>0.88 ± 0.19</td>
<td>1.45 ± 0.31</td>
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</tbody>
</table>

### Table II. Activities of marker enzymes in microsomes and PMs

Membranes were isolated from Avena roots. ATPase activity was determined at an ATP concentration of 5 × 10⁻⁹ M. Activities are the means (±sD) of at least four isolates, with two independent experiments for each isolate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microsome Activity</th>
<th>PM Activity</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol min⁻¹ mg⁻¹ of protein</td>
<td>%</td>
<td>μmol min⁻¹ mg⁻¹ of protein</td>
</tr>
<tr>
<td>Vanadate-sensitive ATPase activity</td>
<td>1.12 ± 0.04</td>
<td>3.01 ± 0.11</td>
<td>2.69</td>
</tr>
<tr>
<td>Glucan-synthase II</td>
<td>6.41 ± 0.56</td>
<td>16.26 ± 1.31</td>
<td>2.54</td>
</tr>
<tr>
<td>Cyt c oxidase</td>
<td>0.85 ± 0.09</td>
<td>0.02 ± 0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>NADH-dependent Cyt c reductase</td>
<td>0.11 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.38</td>
</tr>
<tr>
<td>Latent IDPase activity</td>
<td>0.011 ± 0.002</td>
<td>0.008 ± 0.003</td>
<td>0.68</td>
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</table>
served for latent IDPase activity. In conclusion, the present preparations were sufficiently pure for further analytical research.

**pH Dependency of PM H*-ATPase Activity**

The pH dependency of ATPase activity in both control and FC-treated vesicles was determined at an ATP concentration of 5 mM and in the presence of 0.0125% (w/v) Brij-58. At this concentration ATPase activity in control PM vesicles was maximal at pH 6.5. Increasing the pH of the assay medium to 8.5 resulted in an almost 95% reduction of ATPase activity (Fig. 1). Incubation of the intact root tissue with 10^{-5} M FC prior to PM vesicle isolation (PM + FC) resulted in a stimulation of ATPase activity but only at pH values higher than the optimum pH of ATPase activity in control vesicles (PM - FC) (Fig. 1). At pH 6.5 the treatment with FC resulted in only a minor stimulation (approximately 10%). At pH 7.5 stimulation was 70%, and at pH 8.0 stimulation was at its maximum (approximately 100%).

**Dependency of PM H*-ATPase Activity on the ATP Concentration**

ATPase activity in control and FC-treated vesicles was measured in the ATP concentration range of 5 x 10^{-3} to 5 x 10^{-2} M and at three different pH values: 6.5, 7.0, and 7.5. The concentration dependency was determined in the presence of 0.0125% (w/v) Brij-58. Analysis of the data by curve fitting showed that the concentration dependency of ATPase activity in both types of vesicles and at all three pH values fitted well to a Michaelis-Menten rate equation (Table III). In both types of vesicles an increase of pH reduced V_{max} and K_{m}.

<table>
<thead>
<tr>
<th>pH</th>
<th>V_{max} (µmol min^{-1} mg^{-1} of protein)</th>
<th>K_{m} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>4.52 ± 0.21</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>7.0</td>
<td>2.97 ± 0.06</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>7.5</td>
<td>1.14 ± 0.07</td>
<td>0.21 ± 0.03</td>
</tr>
</tbody>
</table>

K_{m} FC treatment affected only the V_{max} of ATPase activity in vesicles, and no effect was observed on the K_{m}. The effect of FC on the V_{max} was comparable to the pH dependency of ATPase activity (Fig. 1). Increasing the pH resulted in an increase of the stimulatory effect of FC. At pH 6.5 V_{max} was only 4% increased by FC, whereas at pH 7.5 V_{max} was nearly doubled by the FC treatment.

**The Effect of FC on the Amount of PM H*-ATPase**

In both vesicle types a 104-kD protein reacted with the antibody against the central region of the PM H*-ATPase (Fig. 2, lanes C). The immunostained bands in both types of PM vesicles were quantified densitometrically from the western blots. FC treatment had no effect on the amount of PM H*-ATPase. With the amount in control vesicles set at 100%, the amount in FC-treated vesicles was 103 ± 4%.

**The Effect of Trypsin Treatment on PM H*-ATPase Activity**

In control vesicles trypsin treatment resulted in a maximal effect after an incubation of 45 min. This period was subsequently used. The effect of trypsin on ATPase activity was studied in the pH range 5.5 to 8.5 (Fig. 3). In FC-treated vesicles the trypsin treatment did not affect ATPase activity. In control vesicles the trypsin treatment resulted in a stimulation of ATPase activity at pH > 6.5. This stimulation was similar to the stimulation by FC in these experiments (Fig. 4).
Modulation of Plasma Membrane H\(^+\)-ATPase Activity by Fusicoccin

The effect of trypsin treatment on the pH dependency of ATPase activity in PMs from Avena roots. PMs were isolated from control roots (PM - FC; ○, ○) and from roots treated with 10\(^{-5}\) M FC prior to the PM isolation (PM + FC; ■, □). PM vesicles were incubated with trypsin for 45 min (○, □) in the presence of 0.04% (w/v) Brij-58. In control experiments both trypsin and trypsin inhibitor were omitted or trypsin inhibitor was added before the PMs were added. No difference was observed between the two; therefore, only the control without trypsin and trypsin inhibitor is shown (○, ■). ATPase activity was determined at an ATP concentration of 5 \(\times\) 10\(^{-5}\) M and in the presence of 0.0125% (w/v) Brij-58. The curves drawn were computed from the parameters given in Table IV and using Equation 4.

Both FC- and trypsin-sensitive ATPase activities were maximal at pH 7.0.

The trypsin treatment resulted in the appearance of a 97-kD protein in both control and FC-treated vesicles (Fig. 2, lanes T). This protein also reacted with the antibody against the central region of the PM H\(^+\)-ATPase. Apparently the trypsin treatment removed an approximately 7-kD piece from ATPase polypeptides in PM - FC and PM + FC.

The Effect of Lyso-PC on the pH Dependency of PM H\(^+\)-ATPase Activity

The study of the effect of lyso-PC on PM H\(^+\)-ATPase activity was complicated by the fact that at high lyso-PC/protein ratios lyso-PC inhibited ATPase activity at alkaline pH values. At pH values between 7.5 and 8.5 the addition of 0.0125% (w/v) lyso-PC, which corresponds to a lyso-PC/protein ratio of 31.3, inhibited ATPase activity by 80 to 100% when compared with activity in the presence of 0.0125% (w/v) Brij-58. However, at pH values < 7.5 ATPase activity was stimulated by approximately 25%.

Because of the inhibition, the effect of lyso-PC was studied at a lower concentration of lyso-PC (0.001% [w/v], equivalent to a lyso-PC/protein ratio of 2.5). The effect of this lower concentration was determined in the presence of 0.0125% (w/v) Brij-58 because 0.001% (w/v) lyso-PC alone was unable to open the vesicles (data not shown). In the presence of 0.001% (w/v) lyso-PC PM H\(^+\)-ATPase activity was stimulated over a broader pH range (Fig. 5), although stimulation was not as pronounced as in the presence of 0.0125% (w/v) lyso-PC. At pH values of less than 7.0 stimulation was approximately 20% for all pH values studied in both control and FC-treated vesicles. Only small inhibitory effects were observed at pH values 8.0 and 8.5 (20 and 30%, respectively). However, at pH 7.0 lyso-PC was capable of stimulating PM H\(^+\)-ATPase activity in both control and FC-treated vesicles (Fig. 5).

DISCUSSION

Stimulation of PM H\(^+\)-ATPase Activity by FC

Oat roots are a suitable source for PMs, PM H\(^+\)-ATPase activity, and other PM-bound proteins (Sandstrom et al., 1987; De Boer et al., 1989). In the present study the various marker assays showed that contamination of the PM fraction...
and glucan synthase result from the use of a microsomal richments obtained for vanadate-sensitive ATPase activity in PMs from Avena roots. PMs were isolated from control fraction already quite rich in PMs. In the microsomal fraction computed from the parameters given in Table 1282 therefbr, also result from the presence of fluoride in the vitro stimulation in the experiments presented here might, concentrations of fluoride (De Boer et al., 1993). Lack of in PM H+-ATPase is lost during the isolation of the PMs recently that binding of FC to FCBP is inhibited by millimolar to the PM isolation (PM isolation) and from roots treated with 10^-5 M FC prior to the PM isolation (PM isolation). ATPase activity was assayed in the presence of 0.0125% (w/v) Brij-58 (O, □) or 0.001% (w/v) lyso-PC and 0.0125% (w/v) Brij-58 (O, □). The curves drawn were computed from the parameters given in Table IV and using Equation 4.

The Molecular Mechanism of FC Action

Palmgren et al. (1991) demonstrated that the C terminus of the PM H^+-ATPase polypeptide contains an autoinhibitory domain. Removal of this domain by a mild protease treatment resulted in the release of the constraint on the ATPase activity (Palmgren et al., 1990). Stimulation by lyso-PC apparently resulted from interaction between lyso-PC and the C terminus (Palmgren et al., 1991).

In control and FC-treated vesicles a polypeptide with a mol wt of 104,000 was detected by antibodies against the central domain of the PM H^+-ATPase (Fig. 2). Untreated, both types of vesicles showed the same polypeptide pattern on western blots, whereas in both types the trypsin treatment caused a similar change in the polypeptide pattern (Fig. 2). Hence, FC did not protect the PM H^+-ATPase against proteolysis by trypsin, nor did it activate the PM H^+-ATPase by proteolysis, as also observed by Johansson et al. (1993) and Rasi-Cal
dogno et al. (1993). The observed polypeptide patterns before and after trypsin treatment were highly comparable with the ones observed in the same material by Palmgren et al. (1990, 1991). Thus, trypsin also seemed to remove the C terminus in the experiments presented here.

Trypsin treatment affected ATPase activity only in control vesicles (Figs. 3 and 4), and this stimulation was highly comparable with the stimulation by FC in these experiments (Fig. 4). In FC-treated vesicles stimulation by trypsin was completely absent (Figs. 3 and 4). This means that trypsin and FC stimulation are interchangeable and not additive. Combined with the results of the western blots, this means that FC stimulation results from displacement of the C ter-

minus of the ATPase.

Recently, comparable results concerning the mechanism of FC action were presented by De Michielis et al. (1992) and Rasi-Cal
dogno et al. (1993), who both used Rephasus seedlings, and Johansson et al. (1993), who used Spinacia leaves. They reached the conclusion that there is a role for the C terminus in the signal transduction chain between FC, a primary exogenous stimulus, and the PM H^+-ATPase, the final effector. The results obtained from Avena roots presented here also indicate such a role for the C terminus. Involvement of the C terminus in activation by FC, therefore, seems to be a general mechanism in the regulation of the PM H^+-ATPase activity in plant cells of shoots and roots.

For FC action several mechanisms causing displacement of the C terminus seem possible (Palmgren et al., 1991; Johans-
son et al., 1993; Rasi-Cal
dogno et al., 1993). However, one should keep in mind that the displacement by FC is persistent. As demonstrated in this study, activation of the PM H^+-ATPase by FC is not lost during PM isolation. Cocucci and Marrè (1991) demonstrated co-sedimentation of purified FC-binding activity with PM H^+-ATPase activity. Their result also suggests a persistent and a possibly direct interaction of the FCBP with the C terminus of the PM H^+-ATPase.

Figure 5. The effect of lyso-PC on the pH dependency of ATPase activity in PMs from Avena roots. PMs were isolated from control roots (PM - FC; O, □) and from roots treated with 10^-5 M FC prior to the PM isolation (PM + FC; ■, □). ATPase activity was assayed in the presence of 0.0125% (w/v) Brij-58 (O, □) or 0.001% (w/v) lyso-PC and 0.0125% (w/v) Brij-58 (O, □). The curves drawn were computed from the parameters given in Table IV and using Equation 4.

by other membranes was minimal. The seemingly low enrichments obtained for vanadate-sensitive ATPase activity and glucan II synthase result from the use of a microsomal fraction already quite rich in PMs. In the microsomal fraction the vanadate-inhibited fraction of ATPase activity already amounted to 60%.

In vitro FC caused only a small stimulation of the PM H^+-ATPase activity. At pH 7.5 10^-5 M FC gave a stimulation of 20% after 30 min of incubation (data not shown). Apparently part of the signal transduction chain between the FCBP and the PM H^+-ATPase is lost during the isolation of the PMs (Schulz et al., 1990). The FCBP appears to be especially susceptible to inactivation (Aducci et al., 1984). It was shown recently that binding of FC to FCBP is inhibited by millimolar concentrations of fluoride (De Boer et al., 1993). Lack of in vitro stimulation in the experiments presented here might, therefore, also result from the presence of fluoride in the isolation medium. On the other hand, incubation with FC prior to the PM isolation resulted in a significant stimulation, especially at pH values alkaline to the optimum pH for ATP hydrolysis in control vesicles (Fig. 1 and Table III). This confirms the results of Rasi-Caldogno et al. (1986, 1993), Schulz et al. (1990), and De Michielis et al. (1991).
Kinetics of Native and FC-Stimulated ATPase Activity

The pH dependency of ATP hydrolysis by the PM H⁺-ATPase results from the effect of pH on the rate constant of dephosphorylation ($k_{\text{deph}}$) and on the steady-state level of the phosphorylated intermediate (Briskin, 1986, 1990). Because dephosphorylation depends on deprotonation, the effect of pH on $k_{\text{deph}}$ can be given by:

$$k_{\text{deph}} = k_{\text{deph,0}} \cdot \frac{1}{1 + 10^{(pK_{\text{1}} - \text{pH})}}$$  (1)

$k_{\text{deph,0}}$ is the intrinsic rate constant for the unprotonated phosphorylated intermediate, and $pK_1$ is the dissociation constant of the group that is deprotonated and affects dephosphorylation. On the other hand, formation of phosphorylated intermediate ($EP$) depends on protonation of a certain group (Briskin, 1986, 1990). Therefore, the amount of phosphorylated intermediate ($EP$) can be described by:

$$EP = EP_0 \cdot \frac{1}{1 + 10^{(pK_{\text{2}} - \text{pH})}}$$  (2)

in which $EP_0$ is the amount of $EP$ when the relevant group is fully protonated, and $pK_2$ is the dissociation constant of this group.

ATPase activity can be represented by Equation 3 (Amory et al., 1980; Briskin, 1986; Serrano, 1990)

$$v = k_{\text{deph}} \cdot EP$$  (3)

and substituting Equations 1 and 2 in Equation 3 results in

$$v = k_{\text{deph,0}} \cdot EP_0 \cdot \frac{1}{1 + 10^{(pK_{\text{1}} - \text{pH})}} \cdot \frac{1}{1 + 10^{(pK_{\text{2}} - \text{pH})}}$$  (4)

Fitting Equation 4 to the pH dependency of ATPase activity at the various experimental conditions produced the parameters presented in Table IV.

In control vesicles without lyso-PC the obtained values for $pK_1$ (5.6) and $pK_2$ (7.0) are comparable with the ones usually associated with the pH dependency of ATPase activity (Serrano, 1990). FC treatment resulted in a shift of $pK_2$ to a more alkaline value, whereas this treatment did not affect $pK_1$ and the product of $k_{\text{deph,0}}$ and $EP_0$ (Table IV). This means that FC shifts the pH dependency curve of $EP$ formation to more alkaline values and, thus, stimulates the formation of $EP$ at pH values above the optimum pH of the control enzyme. This effect of FC, then, explains the characteristic pattern of its stimulation completely. Based on these results it is attractive to speculate that FC action increases the affinity of the PM H⁺-ATPase for substrate protons at the cytoplasmic side. Because binding of these protons (and of ATP) must precede phosphorylation of the PM H⁺-ATPase (Briskin, 1990), the increased affinity for these protons will, therefore, result in increased levels of $EP$ at cytosolic pH and, consequently, in increased ATPase activity.

Hager et al. (1991) demonstrated with immunooquantification that FC does not affect the amount of PM H⁺-ATPase in the PM, as was confirmed in this study and in the study by Johansson et al. (1993). Kinetic analysis revealed that FC treatment did not affect the product of $k_{\text{deph,0}}$ and $EP_0$ (Table IV), which suggests that $EP_0$ is probably not affected. This indicates that the total amount of PM H⁺-ATPase is not affected. Therefore, the kinetic analysis is in agreement with the results obtained with immunooquantification.

Trypsin treatment affected only ATPase activity in control vesicles (Figs. 3 and 4). Fitting Equation 4 to the pH dependency of trypsin-treated vesicles revealed that trypsin affected the pH dependency in a manner comparable with FC (Table IV). It shifted $pK_2$ to a more alkaline value, without affecting $pK_1$ and the product of $k_{\text{deph,0}}$ and $EP_0$. Therefore, although the effect on $pK_2$ is not as pronounced as in FC treatment, the mechanism of trypsin activation is highly comparable with that of FC activation.

The study of the effect of lyso-PC on ATPase activity was complicated by the fact that at high lyso-PC/protein ratios lyso-PC inhibited ATPase activity at alkaline pH values. This inhibition was also observed by Pedchenko et al. (1990), who used lyso-PC/protein ratios of 5 to 12.5. A distinct lyso-PC

<table>
<thead>
<tr>
<th>Data Source</th>
<th>Vesicle Type</th>
<th>Treatment</th>
<th>$k_{\text{deph,0}} \cdot EP_0$ (nmol min⁻¹ mg⁻¹ of protein)</th>
<th>$pK_1$</th>
<th>$pK_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>−FC</td>
<td></td>
<td>5.03 ± 0.20</td>
<td>5.56 ± 0.05</td>
<td>7.03 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>+FC</td>
<td></td>
<td>4.86 ± 0.17</td>
<td>5.60 ± 0.05</td>
<td>7.32 ± 0.04</td>
</tr>
<tr>
<td>Figure 3</td>
<td>−FC</td>
<td>−Trypsin</td>
<td>4.18 ± 0.40</td>
<td>5.53 ± 0.12</td>
<td>7.14 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>+Trypsin</td>
<td></td>
<td>4.46 ± 0.38</td>
<td>5.60 ± 0.11</td>
<td>7.31 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>−Trypsin</td>
<td>+Trypsin</td>
<td>4.43 ± 0.28</td>
<td>5.57 ± 0.09</td>
<td>7.37 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>+Trypsin</td>
<td></td>
<td>4.69 ± 0.32</td>
<td>5.63 ± 0.09</td>
<td>7.31 ± 0.08</td>
</tr>
<tr>
<td>Figure 5</td>
<td>−FC</td>
<td>−Lyso-PC</td>
<td>5.54 ± 0.25</td>
<td>5.37 ± 0.06</td>
<td>7.09 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>+Lyso-PC</td>
<td></td>
<td>7.30 ± 0.44</td>
<td>5.54 ± 0.09</td>
<td>6.99 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>−Lyso-PC</td>
<td>+Lyso-PC</td>
<td>5.60 ± 0.30</td>
<td>5.37 ± 0.09</td>
<td>7.33 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>+Lyso-PC</td>
<td></td>
<td>7.18 ± 0.50</td>
<td>5.46 ± 0.09</td>
<td>7.20 ± 0.07</td>
</tr>
</tbody>
</table>
stimulation was observed at a low lyso-PC/protein ratio (Fig. 5), although an additional small inhibitory effect of lyso-PC at high pH values could not be excluded. At this low ratio stimulation by lyso-PC appeared to be different from the stimulation by FC or trypsin. Kinetics analysis revealed that without significant effects on $k_{\text{dep},0}$ and $k_{\text{p}}$, lyso-PC increased the product of $k_{\text{dep},0}$ and $E_P$ (Table IV). This result was also obtained when the data for the effect of lyso-PC on the pH dependency of ATPase activity from Palmgren et al. (1988) were fitted to Equation 4 (Table V). Their data sets showed no indication of inhibition by lyso-PC; they used a lyso-PC/protein ratio of 1. However, in their case the increase of the product is the result of the combined effect of demasking latency in stimulation by lyso-PC.

The stimulation by lyso-PC in Avena roots reported in this study, approximately 25%, differed from that obtained by Palmgren and Sommarin (1989), approximately 100% (Tables IV and V) in a latency-free system. It is known that the nutritional status of Avena roots affects ATPase activity (Kuijper et al., 1974). In roots grown on a low-concentration salt medium, like the one used in this study, ATPase activity is increased when compared with roots grown on a high-concentration salt medium, like the one used by Palmgren et al. (1988). The difference in lyso-PC stimulation mentioned above likely resulted from the difference in native ATPase activity. This would imply that the extra ATPase activity induced by the low-concentration salt condition is less sensitive to lyso-PC, FC, and a mild trypsin treatment. This is a plausible explanation, since the isoforms of the Arabidopsis PM H⁺-ATPase expressed in yeast have different sensitivities to lyso-PC (Palmgren and Christensen, 1994). Studies of the regulation of the PM H⁺-ATPase are, therefore, better performed with Avena roots grown in a high-concentration salt medium. Differences in growth conditions might also explain the discrepancies between the relative stimulation by FC in this study and those observed by Johansson et al. (1993) and Rasi-Caldegno et al. (1993), although a comparison between different plant species and tissues may also involve other factors.

It is interesting that fitting the data presented by Palmgren et al. (1988) of the effects of linolenic and arachidonic acid on ATPase activity (Table V) revealed that activation by these fatty acids is comparable to activation by FC (Table IV).

It has been suggested that phospholipids are a requirement for dephosphorylation of the phosphorylated intermediate (Serrano, 1990). The addition of phospholipids induces dephosphorylation and only slightly reduces the steady-state level of phosphorylated intermediate (Vara and Serrano, 1983). Consequently, the increase of the product of $k_{\text{dep},0}$ and $E_P$ might result from an effect of lyso-PC on $k_{\text{dep},0}$.

An important conclusion from this analysis is that activation by FC and trypsin are not comparable with the activation by lyso-PC. Lyso-PC is, thus, probably not the intermediate in the signal transduction chain between FCBP and the PM H⁺-ATPase, which ultimately interacts with the PM H⁺-ATPase and regulates its activity. Also, a comparison between the mechanisms of activation by FC and trypsin, on the one hand, and stimulation by lyso-PC, on the other hand, indicates that they are of a different nature.

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### Table V. Parameters of pH dependency of ATPase activity in PM from Avena roots (data from fig. 2 in Palmgren et al. (1988))

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$k_{\text{dep},0}$</th>
<th>$k_{\text{p}}$, $k_{\text{p}}$</th>
<th>$E_P$</th>
<th>$pK_1$</th>
<th>$pK_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.41 ± 0.12</td>
<td>5.73 ± 0.09</td>
<td>6.69 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lyso-PC</td>
<td>3.19 ± 0.16</td>
<td>5.91 ± 0.05</td>
<td>7.04 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>1.28 ± 0.04</td>
<td>5.81 ± 0.04</td>
<td>7.17 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>1.23 ± 0.03</td>
<td>5.77 ± 0.03</td>
<td>7.29 ± 0.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Modulation of Plasma Membrane H\(^{+}\)-ATPase Activity by Fusicoccin

plasma membrane H\(^{+}\)-ATPase and the fusicoccin receptor from radish microsomes. Plant Sci 73: 45–54


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