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# Proton Transport and Phosphorylation of Tonoplast Polypeptides from Zucchini Are Stimulated by the Phospholipid Platelet-Activating Factor[1]

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## ABSTRACT

**The ether phospholipid platelet-activating factor and certain similar phospholipids, including lysophosphatidylcholine, are known to stimulate both $H^+$ transport and protein phosphorylation in plant microsomal membranes. In the present work, several polypeptides in highly purified tonoplast membranes from zucchini (*Cucurbita pepo* L.) showed platelet-activating factor-dependent phosphorylation. Comparison of protein phosphorylation in different membrane fractions separated by sucrose step density gradient centrifugation indicated that some of the phosphoproteins were contaminants or were common to several membrane fractions, but platelet-activating factor-dependent phosphorylation of peptides at 30, 53, and perhaps 100 kilodaltons was tonoplast specific. The phosphoprotein of 53 kilodaltons was shown by three different approaches (one- and two-dimensional polyacrylamide gel electrophoresis, western blots, and immunoprecipitation) to cross-react with antibody raised against the B subunit of the tonoplast ATPase from red beet (*Beta vulgaris* L.).**

Although the vacuolar $H^+$-ATPases of plants, animals, and fungi have been receiving increasing attention, much of the current work is focused on their structure and evolution (9, 14, 18) and relatively little is known of their regulation. Increased activity of the enzyme has been observed as an adaptation to salt stress (19) and during adaptation to CAM (27), and regulation is also assumed to occur in the turgor responses of guard cells and other systems.

*In vitro*, proton transport and ATPase activity in a light microsomal fraction from zucchini (*Cucurbita pepo* L.) were shown to be increased in the presence of the ether phospholipid 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine (12, 20, 24). This phospholipid was discovered as a PAF[3] triggering many physiological responses in mammals, including an enhanced influx of calcium ions into platelets and the turnover of phosphorylated phosphatidylinositols (5). In plants, PAF presumably mimicks the action of PAF-like plant phospholipids (12, 24) such as lysophosphatidylcholine. A protein kinase stimulated by PAF, lysophospholipids, and $Ca^{2+}$ has been demonstrated in higher plants (12, 21, 23, 24). We proposed that the PAF/lysophospholipid-stimulated protein kinase could be involved in a signal transduction chain participating in cell regulation in higher plants (20–24).

Membrane fractionation studies showed that $H^+$ transport in zucchini microsomes was attributable mostly to the tonoplast (12). The present work, therefore, examines in more detail the phosphorylation of tonoplast proteins and its relationship to the activation of tonoplast $H^+$ transport by PAF.

## MATERIALS AND METHODS

### Plant Material

Zucchini seeds (*Cucurbita pepo* L.) were surface-sterilized with sodium hypochlorite and grown for 4 d in the dark at 28°C on vermiculite. Hypocotyl hooks of about 1 cm were used for membrane preparations.

### Membrane Preparations

The preparation of light microsomes from zucchini has been described (20). Chopped hypocotyls were degassed in homogenization buffer (1 g:1 mL) containing 4% (v/v) ethanolamine, 0.4 M sodium $\beta$-glycerophosphate, 20 mM EDTA, 5 mM DTE, and 1 mM PMSF titrated with acetic acid to pH 7.5. Tissue was homogenized with mortar and pestle and the homogenate was filtered through a nylon cloth and centrifuged at 4500*g* for 10 min. The supernatant was centrifuged for 30 min in a SW 28 rotor (Beckman) at 25,000 rpm on a sucrose cushion of 12 mL of 0.48 M sucrose in homogenization buffer in which the concentration of EDTA was lowered to 2 mM and DTE was omitted. The microsomal fraction (light microsomes) at the interface was collected with a Pasteur pipette and usually frozen and stored in liquid nitrogen. It contains about 40% tonoplast vesicles (29).

### Tonoplast Preparation by Free-Flow Electrophoresis

The method and the purity of the tonoplast preparation are described elsewhere (13, 25, 29).

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[3] Abbreviations: PAF, platelet-activating factor; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; IEF, isolelectric focusing.

### Membrane Preparation by Step Gradient

The protocol described by Martiny-Baron and Scherer (12) was employed.

### $H^+$ Transport Assay

ATP-dependent $H^+$ transport was measured fluorimetrically (12). Light microsomes were equilibrated on PD10 Sephadex G25 columns in 25 mM Mes/BTP, pH 6.5, and 0.3 M sucrose, and 50 to 100 μg/mL protein was used immediately in the assay. The assay buffer contained 25 mM Mes/BTP, pH 6.5, 0.3 M sucrose, 5 μM quinacrine, 0.1 mM EGTA, 0.2 mM EDTA, 3 mM ATP/BTP, pH 6.5, and 10 mM phosphate/BTP, pH 6.5, in a total volume of 2 mL. PAF was taken from ethanolic stock solution (2 or 4 mg/mL), and final ethanol concentrations were kept equal in each set of experiments. Tests were started by the addition of 20 μL of 0.3 M $MgSO_4$ and uncoupled with 20 μL of 1 M $NH_4Cl$. Protein determination was done according to the method of Bradford (1). Proton transport measurements in the presence of different concentrations of $Ca^{2+}$ were done using mixtures of EGTA and $CaCl_2$ and calculating the amount of free $Ca^{2+}$ with a computer program (2). The mixtures were composed by using concentrations of EGTA/$CaCl_2$ of 1 mM/0 mM, 0.9 mM/0.1 mM, 0.8 mM/0.2 mM, 0.7 mM/0.3 mM, 0.58 mM/0.42 mM, 0.5 mM/0.5 mM, 0.35 mM/0.65 mM, or 0.3 mM/0.7 mM, resulting in concentrations of free $Ca^{2+}$ concentrations of <0.001 μM, 0.41 μM, 1.04 μM, 2.08 μM, 4.80 μM, 9.51 μM, 56.37 μM, and 91.03 μM, respectively.

### Protein Phosphorylation and SDS-PAGE

For protein phosphorylation light microsomes were equilibrated with PD10 Sephadex G25 columns in 0.3 M sucrose and 25 mM Mes/BTP, pH 6.5. The final reaction mix contained 25 mM Mes/BTP, pH 6.5, 0.3 M sucrose, 10 mM $MgSO_4$, 1 mM EGTA, 10 mM phosphate/BTP, 50 μg of microsomal protein, and 1 μM [$\gamma$-$^{32}$P]ATP (1–2 × $10^6$ cpm/assay) in a total volume of 100 μL. The reaction was started by the addition of radioactive ATP and stopped by adding 50 μ L of sample buffer (15% [w/v] SDS, 0.2 M Tris-HCl, pH 6.8, 0.3 M DTT, 10% [v/v] mercaptoethanol, and 40% [w/v] sucrose). After rapid mixing, samples were heated for 5 min at 95°C and centrifuged in a table top centrifuge at 10,000*g* for 15 min. Eighty microliters of the supernatant were subjected to gel electrophoresis. Gel electrophoresis was carried out according to the method of Lämmli (8) on 10% polyacrylamide gels. Gels were stained with Coomassie blue R, dried, and exposed to a Kodak X-Omat x-ray film for autoradiography.

### Two-Dimensional PAGE and Western Blot

Protein phosphorylation was carried out as described above except that the ATP concentration was lowered to 0.1 μM (final) and radioactivity was increased (10 μCi/assay) to increase specific labeling. Phosphorylation was stopped after 10 min by the adding 100 μL of buffer (15% [w/v] SDS, 0.15 M Tris/HCl, pH 6.8, 0.2 M DTT, 10% [v/v] mercaptoethanol, and 10 mM PMSF) and heating the sample for 10 min at 80°C. Proteins were precipitated by adding 800 μL of acetone and incubating for 1 h at −20°C. The protein pellet was washed with 80% acetone in 0.2 M Tris, dried, and resuspended with 20 μL of 10% (v/v) Nonidet P-40, 8 M urea, and 0.5 M Tris and kept overnight at −20°C to dissolve the protein pellet. For IEF, 80 μL of solubilization buffer (9.5 M urea, 2% [v/v] Nonidet P-40, 5% [v/v] mercaptoethanol, and 5% [v/v] ampholines) were added and the sample was centrifuged for 15 min at 100,000*g* in a Beckman airfuge to remove nonsolubilized protein. IEF was carried out according to the method of O'Farrell (16). For the second dimension, tube gels were equilibrated 2 × 10 min in 10% (w/v) SDS, 0.5 M Tris-HCl, pH 6.8, 50 mM DTT, 5% (v/v) mercaptoethanol, and 10% (v/v) glycerol, and SDS-PAGE was carried out as described above. Gels were placed on nitrocellulose paper and blotted for 3 h at 100 V at 4°C. For immunostaining, nitrocellulose blots were incubated with antiserum at a dilution of 1:750 for immunodetection. The preparation of the polyclonal antibody against the 57-kD subunit of red beet tonoplast $H^+$-ATPase has been described (10, 11). Immunodetection was done with anti-rabbit goat immunoglobulin G conjugated to horseradish peroxidase purchased from Bio-Rad.

### Immunoprecipitation

Immunoprecipitation was performed as described in Manolson *et al.* (9). About 40 μg of microsomal protein were incubated in a phosphorylation assay as described above, but with specific radioactivity increased by using 0.1 μM [$\gamma$-$^{32}$P] ATP in a final volume of 50 μL (30 μCi/assay). Phosphorylation was stopped after 2.5 min by adding 50 μL of extraction buffer (8% [w/v] SDS in 100 mM Tris-HCl, pH 7.4) and heating the sample for 5 min at 95°C. For immunoprecipitation, the sample was cooled down to ice temperature. Eight hundred microliters of Triton buffer (2.5% [v/v] Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl, pH 7.4, 5 mM PMSF) and antiserum (dilution 1:500) were added and samples were incubated for at least 3 h at 4°C with gentle shaking. Antibody-antigen aggregates were bound to a formaline-treated cell suspension of *Staphylococcus aureus*, pelleted in a table-top centrifuge, washed five times with wash buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.1% [v/v] Triton X-100, 0.02% [v/v] SDS), and washed again two times with wash buffer without any detergents. For gel electrophoresis, immunoprecipitates were resuspended in a sample buffer of 10% (w/v) SDS, 0.3 M Tris-HCl, pH 6.8, and 10% (v/v) glycerol.

### Densitometry

For quantification of phosphorylation, the relative peak areas were estimated by using a LKB-2222–020 Ultra Scan Xl laser densitometer. Only peak areas from the same exposure of a gel were evaluated and compared by this method at a time for a given experiment. The highest value of peak areas of one gel were set to 1, and all others were calculated relative to this reference value.

## Chemicals

[$\gamma$-$^{32}$P]ATP (5000 Ci/mmol) was purchased from Amersham and diluted with unlabeled ATP to the specific radioactivity as indicated. PAF (1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine) was obtained from Sigma or Novabiochem.

## RESULTS

ATP-dependent proton transport in a light microsomal fraction from zucchini was stimulated by increasing concentrations of PAF (Table I). Because we were interested in the possibility that this effect is mediated by protein phosphorylation, and because $Ca^{2+}$, as well as PAF, can regulate phosphorylation (12, 24), we investigated the effects of both of these factors on $H^+$ transport (Fig. 1). The initial rate of transport showed a broad optimum at about 5 $\mu$M $Ca^{2+}$ (Fig. 1a), whereas the steady-state $\Delta$pH declined with increasing $Ca^{2+}$ concentrations (Fig. 1b). PAF stimulation of both the initial rate and steady-state $\Delta$pH was highest in the presence of EGTA and declined with increasing $Ca^{2+}$ (Fig. 1, a and b). Similar data for the steady-state $\Delta$pH had been obtained previously with higher PAF concentrations (20).

To investigate protein phosphorylation, a highly purified preparation of tonoplast membranes was obtained by density gradient centrifugation followed by free-flow electrophoresis to give 90% homogeneity (13, 25, 29). After protein staining following SDS-PAGE (Fig. 2), the two bands representing the catalytic subunits A and B of the tonoplast ATPase were particularly prominent in this purified preparation. A western blot (Fig. 2) probed with polyclonal antibodies raised against subunit B from red beet (16, 17) confirmed the identity of this subunit, running at 53 kD in this system. Autoradiography of the gel after phosphorylation in the presence of increasing concentrations of PAF (remaining lanes of Fig. 2) showed that the most heavily labeled bands are not attributable to ATPase. However, a relatively weak PAF-stimulated labeling appeared to be associated with the 53-kD band. Certain other polypeptides (*e.g.* 30 kD) also showed an increase in phosphorylation with PAF.

To determine which phosphoprotein might be assigned unambiguously to the tonoplast, phosphorylation patterns and PAF stimulation were compared in soluble proteins and in various membrane fractions separated by sucrose step density gradient centrifugation (Fig. 3). Of the phosphoproteins seen in Figure 2, only the 55- and 30-kD bands, and possibly a 100-kD phosphoprotein, were found exclusively in the tonoplast-enriched fractions 1 and 2 of Figure 3. A phosphoprotein with an apparent mass of 53 kD, also present in the tonoplast-enriched fractions 1 and 2, was found in differing amounts in all membrane fractions. A 120-kD phosphoprotein, several bands around 66 kD, and a 35-kD phosphoprotein were found predominantly in the plasma membrane-enriched fractions 4 and 5.

To further investigate the low level of labeling apparently associated with the ATPase subunit B in Figure 2, phosphorylated microsomal proteins were separated by two-dimensional gel electrophoresis (IEF followed by SDS-PAGE). A western blot probed with the antibody against ATPase subunit B, and the corresponding autoradiogram, are shown in Figure 4. The antibody recognized two spots with apparent molecular masses of about 53 kD and isolectric points of pH 5.6. The corresponding autoradiogram showed a weakly labeled spot at the position of the larger polypeptide, identifying the ATPase B subunit as a phosphoprotein.

Immunoprecipitation of the ATPase subunit B was used as a third approach to confirm the phosphorylation of this subunit and to quantify its stimulation by PAF. One major labeled polypeptide (Fig. 5) with a molecular mass of 53 kD was precipitated by the antibody from phosphorylated mi-

**Table I.** *Stimulation of ATP-Dependent Proton Transport in Zucchini Light Microsomes by PAF*

Proton transport measurements were carried out as described in "Materials and Methods" in the presence of 1 mM EGTA. Initial rate of fluorescence quench is given and values are averages of three experiments (±SD). The PAF/protein ratio was 11.05 ± 1.8 $\mu$g PAF/100 $\mu$g protein with 10 $\mu$g/mL PAF.

| PAF | ATP-Dependent $H^+$ Transport |
|---|---|
| *$\mu$g/mL* | *% Q/min × mg protein* |
| 0 | 5.27 ± 0.45 |
| 1 | 6.37 ± 0.55 |
| 5 | 8.73 ± 0.42 |
| 10 | 11.80 ± 0.30 |

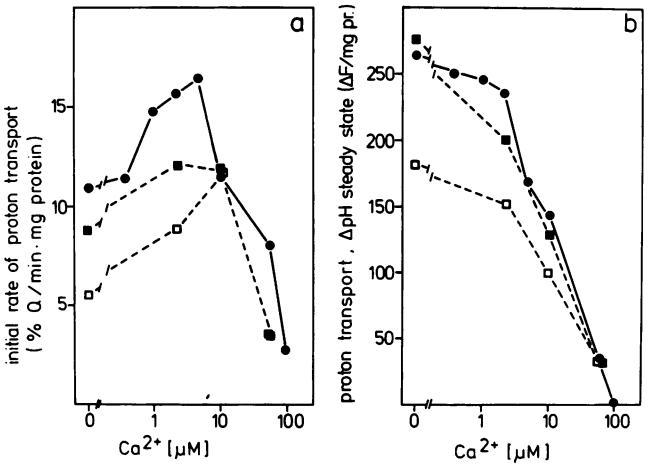



**Figure 1.** Effect of $Ca^{2+}$ and PAF on the initial rate of ATP-dependent proton transport measured as %Q = percent fluorescence quench per minute (a) and as steady-state $\Delta$pH (units in $\Delta F$ = $\%Q(100\% - \%Q)^{-1}$, see ref. 12) in a light microsomal fraction from zucchini hypocotyls. Concentrations of free $Ca^{2+}$ were prepared with EGTA/$CaCl_2$ mixtures as described in "Materials and Methods." To obtain correct values, only assays with the same batch of membranes were compared. This limited the number of assays to eight in one series of measurements so that the experiment comparing the presence and absence of PAF (experiment 2) contained twice as many $Ca^{2+}$ concentrations. One of three sets of experiments is shown. Experiment 1: Effect of a broad concentration range of $Ca^{2+}$ on ATP-dependent proton transport in the absence of PAF (●—●). Experiment 2: The effect of $Ca^{2+}$ on ATP-dependent proton transport in the presence (■—■) and absence (□—□) of 3 $\mu$g/mL PAF. For the comparison, control *versus* PAF, the PAF/protein ratio was 3.75 $\mu$g PAF/100 $\mu$g protein.

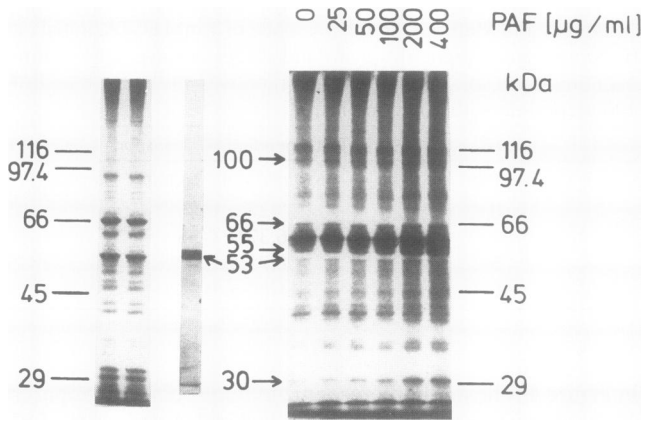



**Figure 2.** PAF-dependent phosphorylation of highly purified tonoplast and comparison of the autoradiography with the immunostaining by an antibody against the B subunit of tonoplast $H^+$-ATPase of red beet root. Tonoplast vesicles were purified to 90% homogeneity as described (25, 29) by free-flow electrophoresis of a microsomal fraction. Each phosphorylation assay contained 300 µg/mL protein, and each lane for electrophoresis contained 24 µg of protein. To the left, two protein-stained lanes with molecular mass markers are shown: in the middle, the immunostained nitrocellulose, and at the right side, the autoradiogram. Arrows indicate the B subunit of the tonoplast $H^+$-ATPase at the 53-kD position and the other PAF-dependent phosphorylated polypeptides mentioned in the text.

crosomal proteins solubilized with SDS extraction buffer. The phosphorylation of this polypeptide was strictly dependent on the presence of $Mg^{2+}$, and there was no nonspecific binding of radioactivity to either the immunoglobulin G or to the formalin-treated *Staphylococcus aureus* cells used to isolate the antibody-antigen aggregate.

Quantification of the immunoprecipitated label is shown in Table II. Calcium ions were found to decrease the extent of the phosphorylation, but PAF stimulated the phosphorylation of the 53-kD polypeptide more than twofold in the presence of EDTA and almost threefold in the presence of calcium ions.

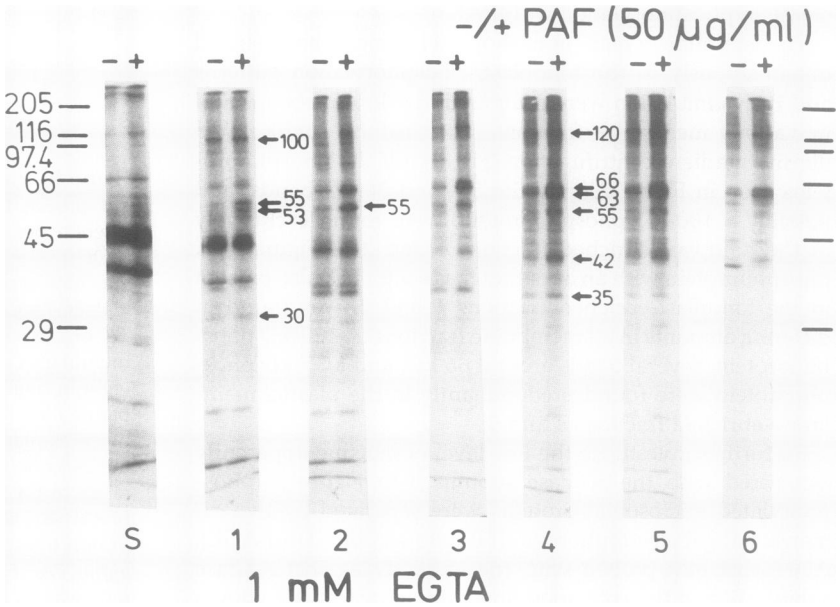
## DISCUSSION

### Stimulation of the Vacuolar ATPase by PAF

The rate of proton transport in a light microsomal fraction from zucchini was doubled in the presence of the highest concentration of PAF (Table I). The transport activity of this fraction is almost exclusively derived from tonoplast $H^+$-ATPase, as shown by fractionation studies (12) and by its complete inhibition by 50 mM nitrate (not shown). Therefore, we can rule out a contribution of the plasma membrane ATPase to the transport stimulation observed here and previously (20). (Stimulation of the nitrate-resistant and orthovanadate-sensitive plasma membrane $H^+$ transport by PAF can, however, be observed using inverted plasma membrane vesicles purified from zucchini hypocotyl by phase partitioning [J. Müller and G. Scherer, unpublished data].)

In principle, stimulation of proton transport can be due either to a direct activation of the ATPase or to a change in ion permeability. However, no change in ion permeability

**Figure 3.** PAF-dependent phosphorylation of soluble and membrane proteins separated by sucrose step gradient. A zucchini hypocotyl homogenate was separated on an isopycnic sucrose step gradient (12). Maximal densities of the fractions were: S, soluble protein; fraction 1, 1.10 g/cm³ (tonoplast-enriched); fraction 2 (ER-enriched), 1.127 g/cm³; fraction 3 (Golgi-enriched), 1.14 g/cm³; fraction 4 (plasma membrane-enriched), 1.56 g/cm³; fraction 5 (plasma membrane-enriched), 1.18 g/cm³; fraction 6 (mitochondria-enriched), 1.22 g/cm³. Marker enzyme data are published (12). Molecular mass markers are indicated. Arrows indicate tonoplast-specific and plasma membrane-specific PAF-dependent phosphoproteins in the respective fractions.



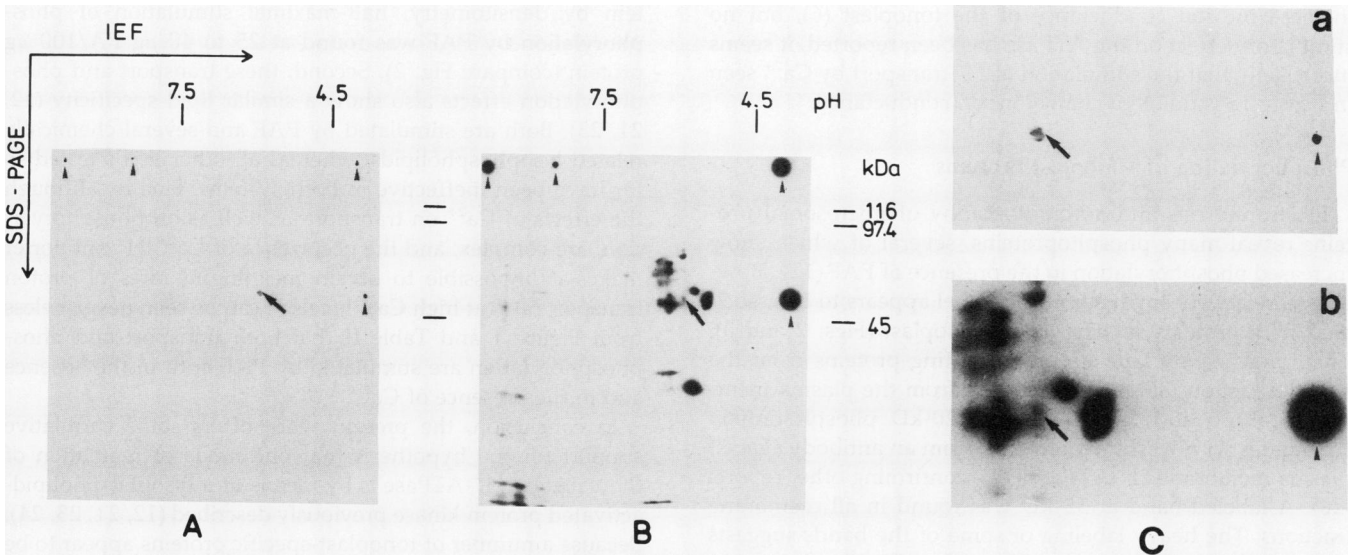



**Figure 4.** Immunodetection of the tonoplast ATPase subunit from zucchini. Zucchini microsomal proteins were phosphorylated in the presence of 100 μg/mL PAF, separated by two-dimensional gel electrophoresis, and transferred to nitrocellulose. The nitrocellulose blot was immunostained with the antibody against the B subunit of red beet tonoplast ATPase. Marks with radioactive ink were made at the sides prior to exposure on an x-ray film and indicated by arrowheads. The position of the immunostained pair of polypeptides and the corresponding label on the autoradiogram is indicated by an arrow. A, Western blot; B, autoradiogram; C, enlarged section of the western blot (C,a) and autoradiogram (C,b).

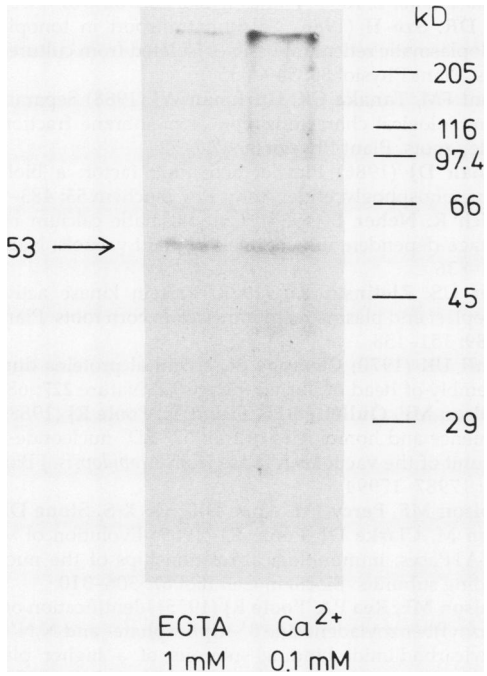



**Figure 5.** Immunoprecipitation of the tonoplast ATPase B subunit from zucchini light microsomes. Proteins were phosphorylated in the presence of 1 mM EGTA or 0.1 mM $CaCl_2$ and 50 μg/mL PAF, and the immunoprecipitate was resuspended in a sample buffer containing no reducing agents prior to gel electrophoresis. The autoradiogram is shown.

could be detected with concentrations of PAF up to 5 μg/mL or about 2.5 μM (24), whereas ATPase activity was stimulated by PAF in the presence of either gramicidin or Triton X-100 (12). Thus, the $H^+$-ATPase enzyme itself is stimulated by PAF.

### Effects of Calcium on Proton Transport

The effects of $Ca^{2+}$ on ATP-dependent transport at the tonoplast were complex. The initial rate of transport showed a broad optimum in the range of 1 to 10 μM $Ca^{2+}$ (Fig. 1a), whereas the steady-state ΔpH steadily decreased with $Ca^{2+}$ concentration (Fig. 1b). The inhibitory effects of $Ca^{2+}$ can be explained by the activity of a $Ca^{2+}/H^+$ antiporter (3), whereas the increase in the initial rate of $H^+$ transport could be due to an increase in anion conductance and/or to a direct stimulation of the ATPase enzyme. Because $Ca^{2+}$ is known to

**Table II.** *Extent of Phosphorylation of the 53-kD Tonoplast Subunit in the Presence and Absence of PAF and $Ca^{2+}$ Determined by Immunoprecipitation*

Quantification was done by laser scan densitometry of relative peak areas of autoradiograms with the highest values set as 1.0, as described in "Materials and Methods." Values are means of two independent experiments and did not differ more than 5% from the average.

| | Addition | |
|---|---|---|
| | EGTA (1 mM) | $Ca^{2+}$ (0.1 mM) |
| Control | 0.44 | 0.20 |
| + 50 μg/mL PAF | 1.0 | 0.67 |

increase the ion conductance of the tonoplast (6), but no stimulatory effect on the ATPase has been reported, it seems more likely that the stimulation of $H^+$ transport by $Ca^{2+}$ seen in Figure 1a is due to a change in ion conductance.

### Phosphorylation of Tonoplast Proteins

Electrophoresis and autoradiography of microsomal proteins reveal many phosphoproteins, several of which show increased phosphorylation in the presence of PAF (12). However, a relatively small amount of label appears to be associated with proteins specific to the tonoplast (Figs. 2 and 3). There are heavily labeled contaminating proteins from the soluble fraction (35 and 45 kD) and from the plasma membrane (63/66 and 120 kD). The 120-kD phosphoprotein comigrates with western blot signal from an antibody against plasma membrane $H^+$-ATPase (15), confirming other reports (28). A labeled band at 55 kD was found in all membrane fractions. The heavy labeling of some of the bands suggests the possibility that they may be autophosphorylating protein kinases (26). If so, it may be understandable that, in comparison, other phosphoproteins carry little label (7).

Of the phosphoproteins that appear to be specific to the tonoplast, the weakly labeled 53-kD band was identified by three different approaches (one- and two-dimensional electrophoresis and immunoprecipitation; Figs. 2, 3, and 4) as subunit B of the tonoplast $H^+$-ATPase. The two-dimensional electrophoresis showed a pair of polypeptides cross-reacting with the antibody to subunit B, which corresponds to a previous observation by Dupont *et al.* (4). Other phosphoproteins that appear to be specific to the tonoplast include polypeptides at 30 and 100 kD (Figs. 2 and 3; see also ref. 15 for the 100-kD phosphoprotein). These may or may not also be subunits of the ATPase (14). All of these tonoplast-specific polypeptides appear to be more heavily labeled in the presence of PAF (Figs. 2 and 3; Table II; ref. 15), and are therefore candidates in a putative signal pathway whereby PAF stimulates vacuolar $H^+$ transport.

Calcium, both with and without PAF, was found to decrease the extent of phosphorylation of the subunit B (Table II). Because of the complex effects of $Ca^{2+}$, the significance is hard to evaluate. In any case, the result contrasts with a report of Zocchi (30) that calcium-increased phosphorylation of corn microsomes was thought to be responsible for a decrease in $H^+$-ATPase activity. However, in the latter work, no attempt was made to distinguish the tonoplast-specific phosphorylation from phosphorylation occurring in other membranes present in these microsomes.

### Correlation of $H^+$ Transport and Protein Phosphorylation

It is seen above that both $H^+$ transport at the tonoplast and the phosphorylation of tonoplast proteins are stimulated by PAF. Based on PAF/protein ratios in microsomal membranes, these two effects require a roughly similar concentration range of PAF. Assuming Michaelis-Menten kinetics for the stimulatory action of PAF (using the values contained in Table I), half-maximal stimulation for proton transport is found at about 10 $\mu$g PAF/100 $\mu$g protein. Using relative phosphorylation values obtained for the 30-kD phosphoprotein by densitometry, half-maximal stimulation of phosphorylation by PAF was found at 25 to 40 $\mu$g PA/100 $\mu$g protein (compare Fig. 2). Second, these transport and phosphorylation effects also show a similar lipid specificity (12, 21, 23). Both are stimulated by PAF and several chemically related lysophospholipids, whereas all other lipids tested so far have been ineffective in both systems. Finally, although the effects of $Ca^{2+}$ on transport, as well as on phosphorylation, are complex, and the occurrence of $Ca^{2+}/H^+$ antiporter makes it impossible to obtain meaningful rates of proton transport rates at high $Ca^{2+}$ levels, it can be seen nevertheless from Figure 1 and Table II that both transport and phosphophorylation are stimulated by PAF both in the absence and in the presence of $Ca^{2+}$.

In conclusion, the present work offers some correlative support for the hypothesis that one mode of regulation of the vacuolar $H^+$-ATPase is by means of a lysophospholipid-activated protein kinase previously described (12, 21, 23, 24). Because a number of tonoplast-specific proteins appear to be phosphorylated by such a kinase, one of which is the ATPase subunit, the significance of phosphorylation for this mode of regulation remains to be determined and warrants further investigations.

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