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A new set of regulatory molecules in plants: A plant phospholipid similar to platelet-activating factor stimulates protein kinase and proton-translocating ATPase in membrane vesicles

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Abstract. 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, an ether phospholipid from mammals known as platelet-activating factor (PAF), specifically stimulates proton transport in zucchini (*Cucurbita pepo* L.) microsomes (G.F.E. Scherer, 1985, Biochem. Biophys. Res. Comm. 133, 1160–1167). When plant lipids were analyzed by two-dimensional thin-layer chromatography a lipid was found with chromatographic properties very similar to the PAF (G.F.E. Scherer and B. Stoffel, 1987, Planta, 172, 127–130). This lipid was isolated from zucchini hypocotyls, red beet root, lupin root, maize seedlings and crude soybean phospholipids. It had biological activity similar to that of the PAF, based on phosphorus content, and stimulated the steady-state ΔpH in zucchini hypocotyl microsomes about twofold. Other phospholipids, monoglyceride, diglyceride, triglyceride, oleic acid, phorbol ester, and 1-O-alkylglycerol did not stimulate proton transport. When microsomes were washed the PAF was ineffective but when soluble protein was added the PAF stimulation of H^+ transport was reconstituted. The soluble protein responsible for the PAF-dependent stimulation of transport activity could be partially purified by diethylaminoethyl Sephacel column chromatography. In the same fractions where the PAF-dependent transport-stimulatory protein was found, a protein kinase was active. This protein kinase was stimulated twofold either by the PAF or by Ca^{2+}. When Ca^{2+} was present the PAF did not stimulate protein-kinase activity. When either the PAF, protein kinase, or both were added to membranes isolated on a linear sucrose gradient, ATPase activity was stimulated up to 30%. Comparison with marker enzymes indicated the possibility that tonoplast and plasma-membrane H^+-ATPase might be stimulated by the PAF and protein kinase. We speculate that a PAF-dependent protein kinase is involved in the regulation of proton transport in plants in vitro and in vivo.

Key words: H^+-ATPase – *Cucurbita* – Plasma membrane – Platelet-activating factor – Phospholipid – Protein kinase, phospholipid-activated – Tonoplast.

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Abbreviations: BTP = 1,3-bis[tris(hydroxymethyl)-methylamino] propane; DEAE = diethylaminoethyl; EGTA = ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid; Mes = 2-(N-morpholino)ethanesulfonic acid; PAF = platelet-activating factor = 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine

Introduction

Platelet-activating factor (PAF) is an unusual phospholipid with an ether linkage to a long-chain alcohol in the C1 position and an ester bond to acetic acid in the C2 position of the glycerol backbone (Demopoulos et al. 1979). It has hormone-like properties in mammals and exerts its effects at nanomolar or lower concentrations (for a recent review see Hanahan 1986). Prominent effects of the PAF in animal cells are a stimulation of Ca^{2+} influx (Lee et al. 1981; Conrad and Rink 1986; Doyle et al. 1986), stimulation of the metabolism of inositol phosphoglycerides (Billah and Lapetina 1983; Fisher et al. 1984), and protein phosphorylation by protein-kinase C (Hanahan and Sugatani 1986). This cascade of events is probably triggered by binding of the PAF to a protein receptor on the cell surface (Hwang et al. 1986).

In plants the PAF stimulates H^+ transport in isolated microsomes (Scherer 1985), and it was found that this activation requires a soluble proteinaceous cofactor (Scherer and Martiny-Baron

1987). This at first appeared to be an artificial system and seemed to be the unexpected property of an unusual lipid. With our observation, however, that the PAF had chromatographic properties very similar to those of an unknown plant phospholipid (Scherer and Stoffel 1987), which affected proton transport in zucchini microsomes in a manner similar to PAF, it became likely that we had discovered a regulatory system indigenous to plants. Since micromolar concentrations of the PAF – and, presumably of the active plant lipid as well – are needed to stimulate proton transport in plant membrane vesicles it seems unlikely that the PAF or the plant lipid have the same hormone-like mechanism of action in plants as the PAF has in animals where nanomolar concentrations are already fully active. A preliminary account of our data has been presented elsewhere (Martiny-Baron and Scherer 1987; Scherer et al. 1987).

Materials and methods

Plant material. Zucchini (*Cucurbita pepo* L., Cocozelle von Tripolis; Schmitz-Laux, Hilden, FRG) seeds were surface sterilized with sodium hypochlorite (1.5%) and grown in moist vermiculite for 4 d in the dark at 25°–30° C to about 3–5 cm height. Hypocotyl hooks about 1 cm long were harvested. Lupin (*Lupinus albus* L., Schmitz-Laux) seeds were soaked in tap water overnight and grown for 4–5 d in moist vermiculite in the dark at 25–30° C to about 5 cm height. Root tips 1–2 cm long were harvested and rinsed in distilled water prior to homogenization. Red-beet (*Beta vulgaris* L.) tap root was purchased locally during the winter season. Maize (*Zea mays* L. cv. 'Mutin', Schmitz, Bonn, FRG) was soaked in tap water for 12 h and grown on moist cotton for 4 d at 25°–30° C in the dark. The mesocotyls, primary leaves, and coleoptiles were harvested.

Membranes. Plant material was chopped finely with a razor blade in 4% ethanolamine (v/v), 20 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithioerythritol (DTE), 0.4 M sodium β-glycerophosphate (grade III; Sigma, Munich FRG), titrated with acetic acid to pH 7.5, and homogenized with a mortar and pestle. During homogenization and all following operations the temperature was kept at 4° C. For zucchini the ratio of buffer to fresh weight was 1:1, for lupin and red beet root 1.5:1. The light microsomes used for most of the proton-transport experiments were prepared as the upper band of a sucrose step gradient (fraction A2 as described in Scherer and Fischer 1985). This upper band of microsomes contained soluble proteins since it overlapped with the supernatant. Washed microsomes were prepared by pelleting them once for 60 min at 25000 rpm in a SW 28 rotor (Beckman, Düsseldorf, FRG) and resuspension in homogenization buffer. Membranes for centrifugation on a linear sucrose gradient were prepared in a way similar to that described by Scherer (1981), and plant material was homogenized in the homogenization buffer as above with additional 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid (EGTA). Soluble proteins and β-glycerol-phosphate, both leading to undesirable acid-phosphatase activity, were removed by chromatography on a Sepharose 2B–CL column with a column buffer of 4% ethanolamine (v/v), 10 mM 1,3-bis[tris(hydroxymethyl)-methylamino] propane (BTP), 2 mM EDTA, 5 mM EGTA, titrated to pH 7.5 with acetic acid. Turbid, membrane-containing fractions were collected and pipetted onto a linear sucrose gradient in 17-ml centrifugation tubes of SW 27.1 rotor (Beckman). Gradients were made by layers of sucrose in column buffer omitting the EGTA (0.5 ml 1.5 M; 0.5 ml 1.4 M; 0.5 ml 1.3 M; 1.0 ml 1.2 M; 1.0 ml 1.15 M; 1.0 ml 1.1 M; 1.0 ml 1.05 M; 1.0 ml 1.0 M; 1.0 ml 0.95 M; 1.0 ml 0.9 M; 0.5 ml 0.8 M; 0.5 ml 0.6 M; 0.5 ml 0.3 M). Gradients were centrifuged for 16–18 h. The density of the fractions after centrifugation was determined by reading the refractive indices and calibrating them with sucrose solutions in buffer of known densities. After fractionation, membranes were frozen in liquid nitrogen and kept at −80° C for further use.

Protein-kinase purification. Protein kinase was purified from zucchini hypocotyls using the procedure of Schäfer et al. (1985). A diethylaminoethyl (DEAE) Sephacel column of 40 ml volume was used for 50 g fresh weight of hypocotyls. A linear gradient of 0 to 0.5 M NaCl and a total volume of 200 ml was applied for elution. Fractions of 4 ml were collected. For tests of ATPase or of H^+-transport activity, NaCl was removed by chromatography on small prepacked Sephadex G25 columns (Pharmacia, Freiburg, FRG) in 10 mM 2-(N-morpholino)ethanesulfonic acid (Mes)/arginine pH 6.5 containing 0.3 M sucrose. In H^+-transport assays, 0.7 ml of a column fraction was added to one test in a total volume of 2.0 ml. For ATPase tests 5 µl or 10 µl were added to a total assay volume of 100 µl.

Assay of H^+ transport. The quinacrine-fluorescence-quench assay for H^+ transport was performed as described previously (Scherer 1985; Scherer and Fischer 1985), except that tests for the biological activity of lipids such as the PAF or others were all carried out in the presence of 0.1 mM EGTA and of 10 mM phosphate-BTP as a permeant anion at pH 6.5. Lipids were added in ethanolic stock solutions so that the ethanol concentrations never exceeded 1%. Controls contained the same amounts of ethanol, and this ethanol concentration either inhibited transport activity only slightly or not at all. Activity was determined by uncoupling in the steady state. Setting the initial fluorescence as 100% Q, units can be obtained using the equation $\Delta F = 100\ Q\ \%\ (100\text{–}Q\%)^{-1}$, Q % being the decrease in fluorescence percentage in the steady state. ΔF is linear with the amount of protein (Gogarten-Boekels et al. 1985; Scherer and Fischer 1985).

Assay of ATPase. For ATPase assays, membrane fractions were chromatographed on small prepacked Sephadex G25 columns in Mes/arginine, pH 6.5, in 0.3 M sucrose. Protein was measured prior to and after Sephadex chromatography, and all activity measurements were corrected for protein content prior to Sephadex chromatography for comparison with other marker-enzyme measurements. Tests contained 50 µl of membranes, 25 µl of 80 mM Mes-BTP to yield the desired assay pH of either 6.5 (tests with the PAF and orthovanadate) or 7.0 (tests with nitrate), and 25 µl of a mixture containing 4 mM $MgSO_4$, 4 mM ATP (BTP salt), 0.4 mM EGTA, 4 mM sodium molybdate, 4 mM sodium azide, and if so indicated, either 200 mM nitrate-BTP pH 7.0 or 2 mM sodium orthovanadate buffered with Mes to pH 6.5. Where indicated, chromatographed protein-kinase fractions – 5 or 10 µl per assay – were added to this mixture. These fractions had no endogenous ATPase activity on its own (data not shown). Either ethanol or the PAF from a 4 $mg \cdot ml^{-1}$ ethanol stock solution was added to the membrane fraction to give a final assay concentration of 10 or 20 $\mu g \cdot ml^{-1}$ PAF. Tests were started with membranes, and incubated for 20–45 min at 25° C, and stopped by the

addition of 0.4 ml ice-cold 10% trichloroacetic acid. Then, 1 ml of phosphate reagent was added. The reagent contained 6 g ascorbate in 120 ml 1% sodium dodecylsulfate to which 3 ml concentrated sulfuric acid and 3 ml 10% ammonium molybdate had been added. Color was developed for 30 min at 37° C and tests kept on ice prior to reading at 750 nm. Acid-catalysed ATP destruction was minimal under these conditions.

Protein-kinase activity. The method described by Kikkawa et al. (1983) was used. To 10 µl of Sephacel-chromatographed protein-kinase fraction, 90 µl of a mixture containing 0.1 mM EGTA, 20 mM tris(hydroxymethyl)aminomethane-HCl pH 7.5, 5 mM $MgCl_2$, 0.8 mg·ml^{-1} histone (H_1), and 25 µM γ-labeled [^{32}P]ATP ($2 \cdot 10^6$–$3 \cdot 10^6$ cpm per assay) was added. Tests were incubated for 15 min at 30° C, stopped with 0.5 ml of ice-cold trichloroacetic acid (10% w/v), collected on nitrocellulose filters, and washed with four 5-ml aliquots of 10% cold trichloroacetic acid. Filters were dried and counted. Fractions with peak activity in the DEAE Sephacel column contained about 1 mg·ml^{-1} protein. When indicated, tests contained various Ca^{2+} concentrations added as $CaCl_2$.

Marker enzymes. Enzyme tests were as described previously (Scherer 1984a; Buckhout 1983).

Lipid isolation. For the isolation of the PAF-like plant lipid, either 0.5 ml 10% (w/v) crude soybean phospholipid (asolectin) in chloroform or a total lipid extract (Bligh and Dyer 1959) of microsomes, isolated from 10 g tissue and concentrated in 3 ml chloroform, was separated on a 20·20 cm^2 silica-gel thin-layer plate in a solvent system of chloroform:methanol:ammonium hydroxide:water = 60:35:7:2.8 (by vol.). Lipids were visualized either with a phosphorus-detecting spray (Skipsky and Barcley 1969) or, for preparative purposes, with water, and the band containing active plant lipid was scraped off and extracted with 2–3 ml chloroform:methanol (1:2, v/v). The extract was dried with a stream of nitrogen and redissolved in a small amount of ethanol (150–200 µl). A portion of this extract was used for the determination of lipid-bound phosphorus (Rouser et al. 1966), and usually 5–20 µl was used to assay H^+ transport. Controls with the same amounts of ethanol and two concentrations of the PAF were also made with the same batch of microsomes since the response to the PAF varied somewhat. Two-dimensional lipid chromatography and determination of the phospholipid composition of microsomes has been described by Scherer and Morré (1978a).

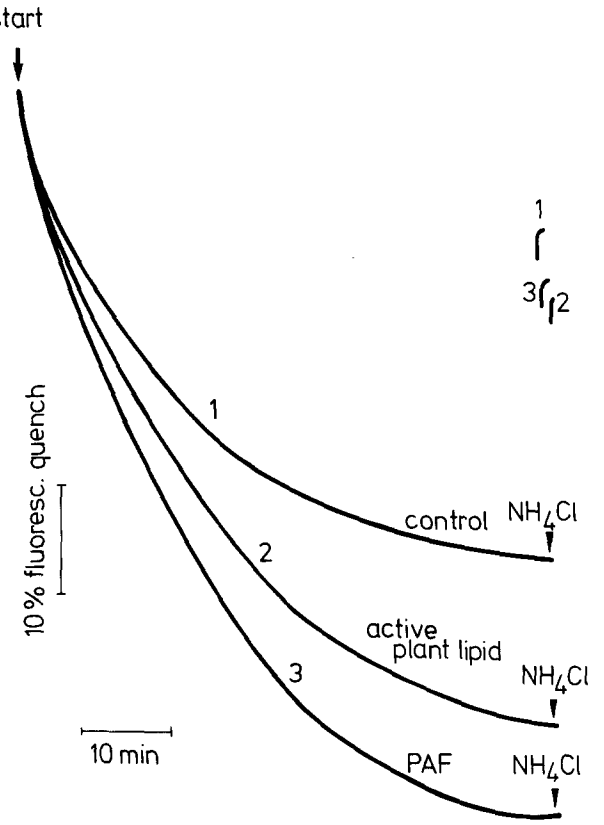

Fig. 1. Stimulation of proton transport in zucchini hypocotyl microsomes by the PAF and by an active plant lipid. Microsomes containing soluble proteins were prepared (=fraction A2 as in Scherer and Fischer 1985) and to the same batch of membranes in three parallel assays was added either ethanol alone or 10 µg·ml^{-1} (=19 µM) PAF or plant lipid in the same amount of ethanol (1 µM lipid-bound phosphorus). Plant lipid had been isolated from crude soybean phospholipids by one-dimensional thin-layer chromatography. Tests were started by the addition of ATP and terminated by uncoupling with 10 mM ammonium chloride. Both lipids increased the steady state ΔpH

Results

The effect of PAF on proton transport in zucchini microsomes has been described previously (Scherer 1985) and is shown here in comparison with the active plant lipid isolated from commercially available crude soybean phospholipids, which has similar biological activity (Fig. 1). As can be seen from the time courses of the three parallel assays, in comparison with the control the PAF and the plant lipid increased the steady-state ΔpH rather than the initial rate of proton transport. We assume that this feature may reflect the fact that the modification reaction mediated by the PAF-dependent protein (see below) needs at least several minutes to become apparent as a change in the activity of proton transport. Therefore, we chose the steady-state ΔpH as a measure of the biological activity of lipids on proton transport.

In principle, proton transport in isolated vesicles can be modified in several ways: H^+-ATPase can be stimulated, the permeability of the vesicle membrane can be altered, or the cotransport of permeable anions can be affected. These questions have been addressed in a previous paper (Scherer and Martiny-Baron 1987). It was found that the PAF stimulates ATP hydrolysis by increasing the V_{max} and decreasing the K_m by 28%. Also, the apparent proton permeability was not changed by 5 µg·ml^{-1} PAF, either in the presence of 10 mM chloride or in the presence of 10 mM phosphate as a permeant anion (Fig. 2). The observed independence of the anion present in in the permeabili-

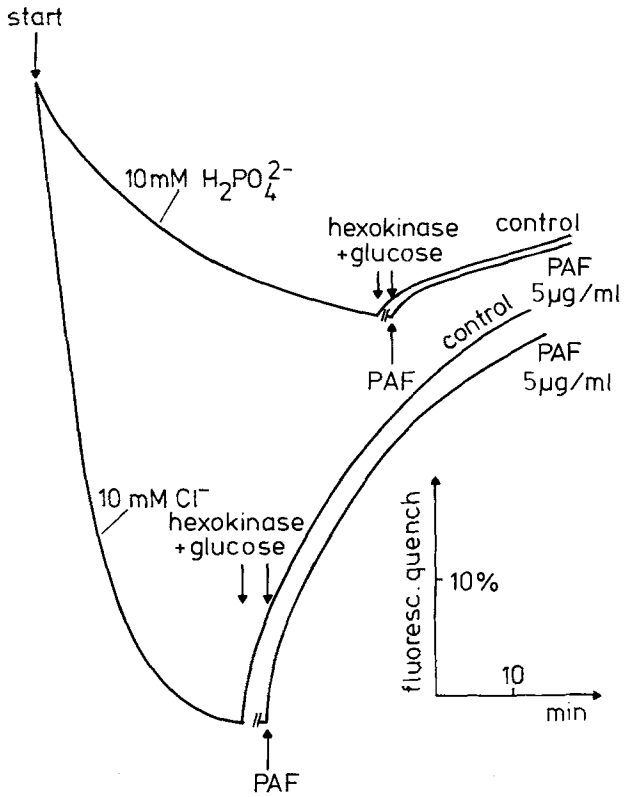

Fig. 2. Effect of the PAF on the apparent passive permeability to protons in zucchini microsomes. In two parallel assays either with 10 mM phosphate-BTP or 10 mM chloride-BTP pH 6.5 a steady-state ΔpH was developed in the vesicles. Then, 25 µl glucose and hexokinase (10 mg·ml^{-1} in 1 M glucose) were added to remove the ATP and immediately thereafter either 5 µl ethanol or an ethanolic solution of 2 mg·ml^{-1} PAF (final concentration: 5 µg·ml^{-1} PAF), and the passive backflow of protons was recorded

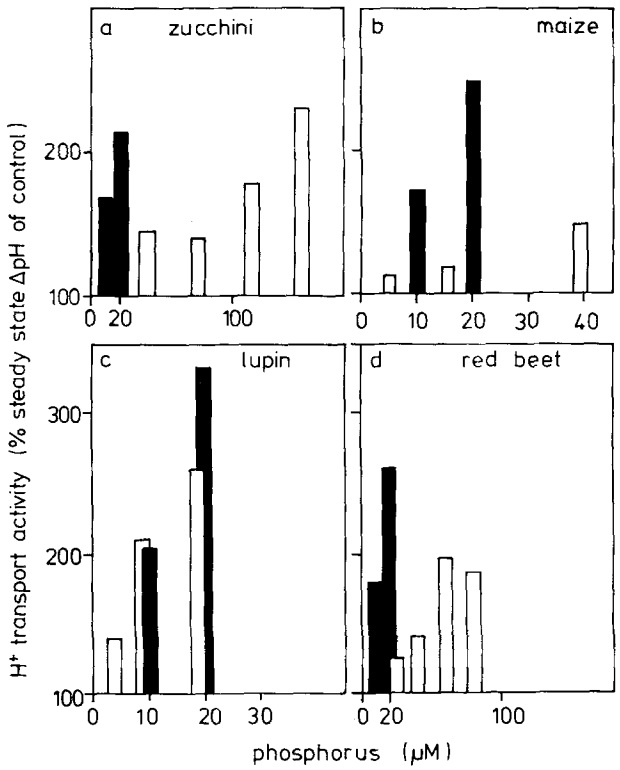

Fig. 3. Stimulation of proton transport in microsomes from various plants. Plant lipid was isolated from total lipid extracts of microsomes by one-dimensional thin-layer chromatography. Phosphorus content and transport stimulatory activity of each isolated batch (*open bars*: lipid from plants as indicated in the panels) was determined and compared with the stimulatory activity of the commercially available PAF (*dark bars*). Proton-transport assays shown in one panel were all done with the same batch of microsomes and were conducted in the presence of 10 mM phosphate-BTP pH 6.5 as a permeant anion. The steady-state ΔpH was determined in units of $\Delta F = 100\ Q\%\ (100\text{–}Q\%)^{-1}$

ty test made it unlikely that anion cotransport is altered by the PAF. Rather it seems likely the the PAF activated the H^+-ATPases.

A PAF-like plant lipid was extracted from several sources, and the biological activity was compared with that of authentic PAF (Fig. 3). Lipid isolated from soybean and lupin roots was equally active, based on lipid-bound phosphorus and assuming only one phosphorus atom per molecule. Similar results had been obtained with lipid from soybean (Fig. 1; see Scherer and Stoffel 1987). A similar chemical identity of the plant lipid and PAF is inferred from the strong chromatographic similarity (Fig. 4). Active plant lipid from soybean and from lupin root had a specific activity comparable to that of the PAF. The relatively low specific biological activity of lipid from zucchini, red beet, and maize could indicate either chemical heterogeneity of the active compound or phospholipid impurities in lipid extracted from these sources.

The chemical similarity of the plant lipid and the PAF is shown by two-dimensional thin-layer chromatography of a total lipid extract from zucchini microsomes (Fig. 4). The thin-layer plates were sprayed with a phosphorus-detecting reagent, which visualized only phospholipids. A comparison of a plate without PAF added and with PAF showed that a spot indicating a minor phospholipid was increased by the addition of PAF to the zucchini lipid extract. Since this spot did not coincide with any of the known standards, we concluded that it contained a PAF-like phospholipid which is responsible for the effect on proton transport (Figs. 1, 3).

In addition to the common phospholipids, we also tested lipids that activate animal protein

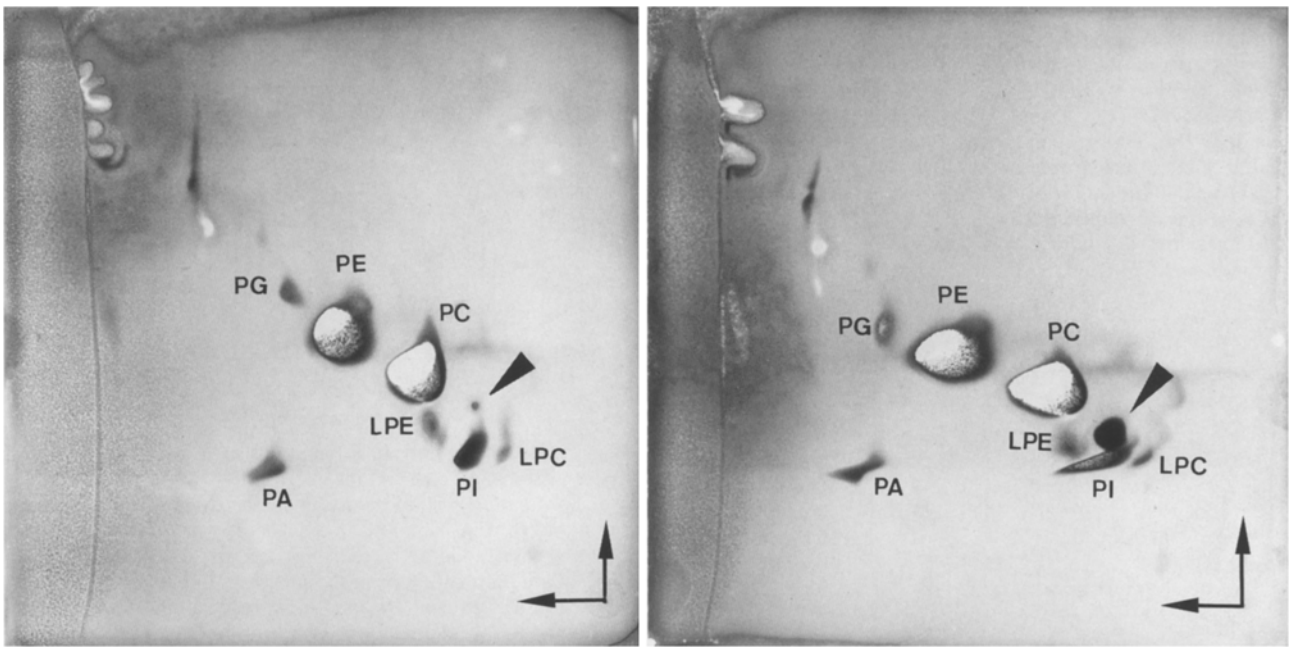

Fig. 4. Two-dimensional thin-layer chromatography of lipids from zucchini microsomes. Total lipids were extracted from zucchini microsomes (Bligh and Dyer 1959) and chromatographed on silica-gel thin-layer plates with a solvent system of chloroform:methanol:ammonium hydroxide:water = 60:35:7:2.8 (by vol.) in the first dimension, and with a solvent system of chloroform:methanol:-acetic acid:acetone:water = 50:10:10:20:5 (by vol.) in the second dimension. Only phospholipids were visualized by spraying the plates with a phosphorus-detecting reagent (Skipsky and Barclay 1969). *Left panel*: zucchini lipids only. *Right panel:* zucchini lipids and 30 µl of 2 mg·ml^{-1} PAF (= 115 nmol, assuming purity) added prior to chromatography. The PAF cochromatographed with a minor zucchini phospholipid which is indicated by an *arrow*. *PA* = phosphatidylinositol; *PC* = phosphatidylcholine; *PE* = phosphatidylethanolamine; *PG* = phosphatidylglycerol; *PI* = phosphatidylinositol; *LPC* = lysophosphatidylcholine; *LPE* = lysophosphatidylethanolamine

kinase C (Takai et al. 1979; Kishimoto et al. 1980; Kaibuchi et al. 1981; Castagna et al. 1982; Kikkawa et al. 1982; Hannun et al. 1986; Ganong et al. 1986). Protein kinase C is activated by phosphatidylserine and diglyceride, both of which decrease greatly its dependence on Ca^{2+}. Phorbol esters replace the natural activator, diglyceride, and overstimulate protein kinase C, leading to tumor growth (Nishizuka 1984). Except for PAF, none of the lipids we tested had appreciable biological activity in the zucchini transport assay (Table 1). Although it is difficult to completely dismiss the possibility that other lipids are effective in our system, these results clearly indicate that the PAF-dependent kinase (see below), which we assume to mediate the regulatory effect of the PAF in plant proton transport, is different from the animal protein kinase C in its activator pattern.

When lipid extracts of zucchini hypocotyl microsomes were analyzed for their content of the biologically active plant lipid, we found that the active lipid made up approx. 1% of the total lipid phosphorus (Table 2), and thus is a minor phospholipid in this tissue. The low amount of this biologically active plant lipid and the fact that it tends to streak into phosphatidylinositol in thin-layer chromatography can easily explain why it may often have been overlooked.

All experiments so far have demonstrated the biological activity of lipids only. The notion that a protein kinase could mediate this effect on proton transport (Scherer 1985) was examined when the effect of the PAF on washed microsomes and on microsomes reconstituted with supernatant proteins was investigated (Fig. 5). This experiment made clear that cytosolic proteins contained a protein factor that was needed for the effect of the PAF on membranes. Bovine serum albumin (Fig. 5) or boiled supernatant (not shown) did not reconstitute the effect of the PAF.

Soluble zucchini proteins were chromatographed on a DEAE Sephacel column under conditions similar to those used for the purification of protein kinase C (Takai et al. 1977; Kikkawa et al. 1982) and also for Ca^{2+}-dependent protein kinase from plants (Schäfer et al. 1985; Elliott and Skinner 1986; Olah and Kiss 1986; Favre and Turian 1987). When the column fractions were tested

Table 1. Stimulation of proton transport by lipids in zucchini microsomes containing cytosolic proteins. Lipids were added from ethanolic stock solutions so that the final ethanol concentration never exceeded 0.5% in the tests. Transport assays were conducted at pH 6.5 with 10 mM phosphate-BTP as anion. To avoid variability, experiments were done with the same batch of membranes. Steady-state ΔpH was determined in duplicate assays as units of $\Delta F = 100$ Q % $(100-Q\ \%)^{-1}$ where Q % is the fluorescence quench in % measured by uncoupling in the steady state which is linear with the amount of protein (Gogarten-Boekels et al. 1985). In controls the ΔF units were set as 100%

	% of control	Concentrations
Experiment A		
Platelet-activating factor	191±22	10 μg·ml^{-1} (=19 μM)
1-O-hexadecylglycerol	111±14	50 μM
1-Monoolein	114±20	10 μg·ml^{-1}
1,2-Diolein	112±10	10 μg·ml^{-1}
Triolein	111±5	10 μg·ml^{-1}
Phorbol-12-myristate-13-acetate	98±17	10 μg·ml^{-1}
Experiment B		
Platelet-activating factor	197±20	10 μg·ml^{-1}
Phosphatidylserine	108± 9	10 μg·ml^{-1}
Phosphatidylinositol	108±12	10 μg·ml^{-1}
Phosphatidic acid	103± 9	10 μg·ml^{-1}
Phosphatidylglycerol	107±11	10 μg·ml^{-1}
Phosphatidylcholine	105± 7	10 μg·ml^{-1}
Phosphatidylethanolamine	107±13	10 μg·ml^{-1}

Table 2. Phospholipid composition of zucchini microsomes. Zucchini microsomes were isolated as described (Scherer 1981) and total lipids extracted from the membranes of the postmitochondrial supernatant (Bligh and Dyer 1959). Two-dimensional thin-layer chromatography was done as described by Scherer and Morré (1978a). Lipids were identified by cochromatography with known standards. Quantitative determination of phospholipids ($n=7$) was as described by Rouser et al. (1966)

Phospholipid	% of total
Phosphatidylcholine	45.3±5.4
Phosphatidylethanolamine	30.4±2.4
Phosphatidylinositol	9.2±1.6
Phosphatidylglycerol	3.3±2.3
Phosphatidic acid	4.8±1.6
Lysophosphatidylcholine	1.5±1.3
Lysophatidylethanolamine	2.2±0.9
N-Acylphosphatidylethanolamine	2.0±2.7
PAF-like plant lipid	1.1±0.8

for their PAF-dependent stimulatory activity in proton-transport assays with washed zucchini microsomes, the fractions eluting with 0.3 M NaCl showed a peak of this activity (Fig. 6).

When protein kinase activity was tested in the fractions of a similar DEAE Sephacel chromatography of soluble zucchini proteins, a peak of kinase activity was found at the same position of the profile as the PAF-dependent proton transport-stimulatory protein had been found (compare Fig. 6 with Fig. 7).

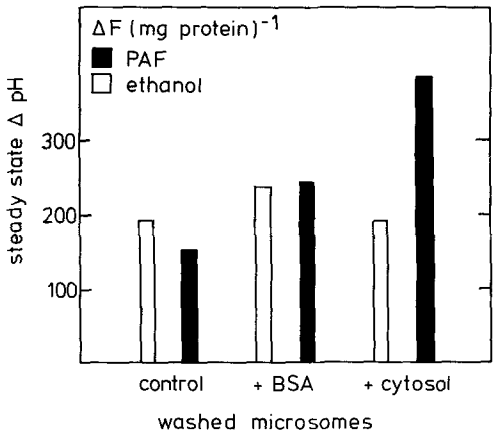

Fig. 5. Reconstitution of the stimulatory effect of the PAF on proton transport in zucchini microsomes by zucchini supernatant proteins. Zucchini microsomes were washed by centrifugation and resuspension and tested for their proton-transport activity either alone or in the presence of 0.5 mg·ml^{-1} bovine serum albumin (BSA) or in the presence of 0.36 mg·ml^{-1} soluble zucchini proteins. Prior to the assay, soluble proteins were prepared by centrifugation of membranes at 100000·*g* for 60 min in the isolation buffer and equilibration in 10 mM Mes-arginine pH 6.5 containing 0.3 M sucrose on small prepacked Sephadex G25 columns. Per assay, 700 μl of soluble protein were added and the concentration is given as the final concentration in the assay of a total volume of 2 ml. Parallel assays containing 10 μg·ml^{-1} PAF (*dark bars*) were conducted (controls: *open bars*). The steady-state ΔpH was determined in proton-transport assays containing 10 mM phosphate-BTP as a permeant anion by uncoupling in units of $\Delta F = 100$ Q % $(100-Q\%)^{-1}$

We further investigated the effect of the PAF and of Ca^{2+} on the partially purified, soluble protein kinase. Using histone as a substrate, the partially purified protein kinase was stimulated twofold by PAF or by Ca^{2+} (Fig. 8). This stimulation by the PAF was only observed in the absence of Ca^{2+} and it was optimal with 15 μg·ml^{-1} PAF. When 100 μM Ca^{2+} was added, protein kinase activity was also stimulated twofold but was no longer PAF-sensitive. Higher concentrations of Ca^{2+} did not stimulate protein kinase activity any further. Stimulation by the PAF but not by other phospholipids or by diglyceride of endogenous protein kinase was also obtained (not shown). The experiments shown in Fig. 6–8 can be explained by the assumption that the soluble protein factor needed for transport stimulation (Fig. 6) could be a phospholipid-stimulated protein kinase (Fig. 7, 8).

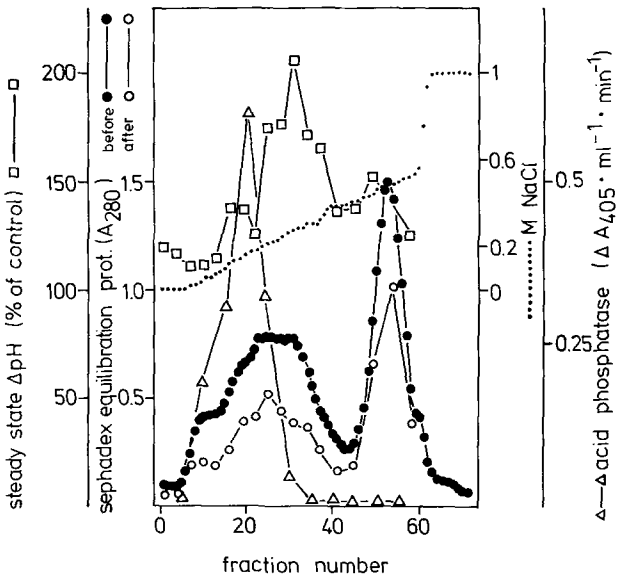

Fig. 6. The PAF-dependent stimulation of proton transport in washed zucchini microsomes by soluble zucchini proteins partially purified on a DEAE Sephacel column. A preparation of soluble proteins from zucchini was bound to DEAE Sephacel and eluted with a linear NaCl gradient (· · · · ·). Optical density at 280 nm was read prior to (●—●) and after (○—○) equilibration on Sephadex G25 columns of the resulting fractions in H^+-transport-assay buffer in order to remove sodium and chloride ions from the soluble proteins. To two parallel transport assays, 0.7 ml equilibrated fraction was added and either ethanol or PAF from ethanolic stock solution (10 $\mu g \cdot ml^{-1}$ final concentration) was added, and the steady-state ΔpH was determined in units of $\Delta F = 100$ Q % $(100\text{–Q }\%)^{-1}$. Transport stimulation is given as % of the control (□—□). All transport assays for one column were done with the same batch of membranes. Acid phosphatase was determined with the fractions containing NaCl (Δ—Δ) and served as an additional marker for comparisons of different experiments

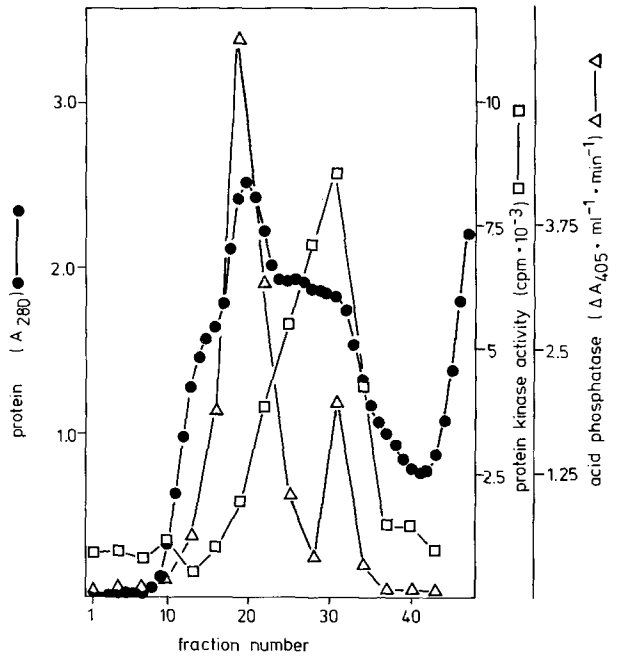

Fig. 7. Partial purification of soluble protein kinase by DEAE-Sephacel column chromatography. A preparation of soluble proteins from zucchini hypocotyl (100000 · *g* supernatant) was bound to DEAE-Sephacel and eluted with a linear NaCl gradient in a manner exactly as in **Fig. 6.** Fractions were read at 280 nm for protein content (●—●), and acid-phosphatase activity was tested (Δ—Δ) to provide an additional marker for the comparison of fractions with **Fig. 6.** Protein kinase activity was determined with histone as the substrate (□—□)

It is known that at least 80% of the proton-transport activity in microsomes is derived from the tonoplast (Scherer and Fischer 1985; vom Dorp et al. 1986). An effect on both plasma-membrane and tonoplast H^+-ATPase had been suggested by transport experiments (Scherer and Martiny-Baron 1987). To further investigate this question, we separated zucchini hypocotyl membranes on a linear sucrose gradient. The PAF and DEAE-purified protein kinase were added separately and together, and the stimulation of ATPase activity was measured and compared with various marker enzymes. Stimulation of ATPase activity by the PAF alone (5 or 10 $\mu g \cdot ml^{-1}$ PAF) was low and predominantly observed in the dense fractions of the gradient. The partially purified protein kinase had no ATPase activity on its own in control experiments (not shown), so that the observed stimulation of ATPase activity must have been a consequence of the interaction of the partially purified protein kinase and membranes. When 10 $\mu g \cdot ml^{-1}$ PAF was added together with the partially purified protein kinase, a further increase of ATPase-activity stimulation was only observed in the plasma-membrane-enriched fractions. In fractions of low density a plateau value of ATPase-activity stimulation was reached by consecutive increases in partially purified protein kinase and-or the PAF concentration. Therefore, we conclude that interaction of the PAF and the protein-kinase-containing column fractions is necessary to bring about stimulation of ATPase activity and of proton transport (Fig. 1, 3).

When we compared the marker-enzyme profiles with the effects of PAF and partially purified protein kinase, the ATPase stimulation in the dense fractions correlated partially with the presence of high activities of uridine 5′-diphosphoglucose (UDPG)-sterolglucosyltransferase (Fig. 9c), a marker for plasma membrane (Hartmann-Bouillon et al. 1979). Vanadate-inhibited ATPase activity coincided only partially with this marker and may not be a useful plasma-membrane marker in all

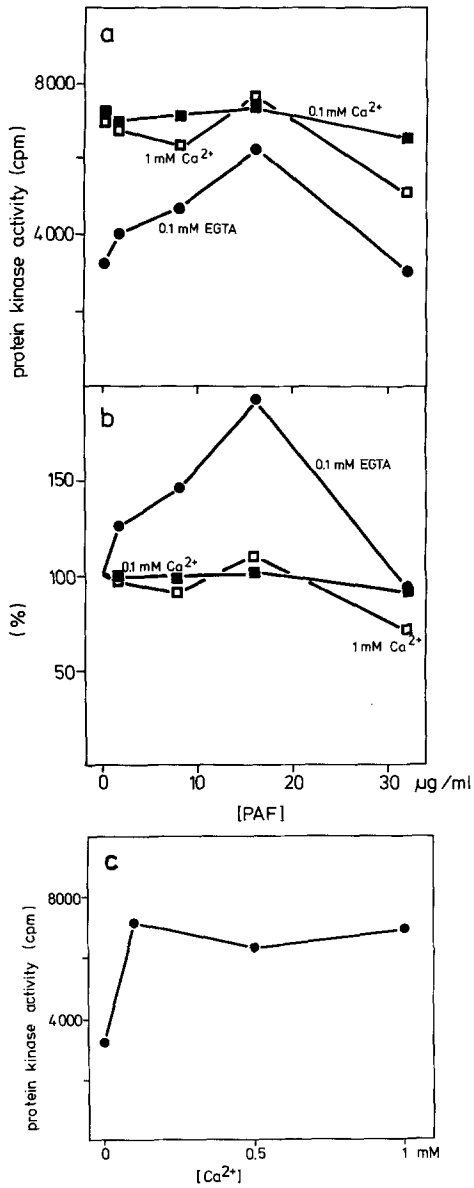

Fig. 8a–c. Dependence on Ca^{2+} and PAF of activation of DEAE-purified soluble protein kinase from zucchini. Protein kinase was isolated by DEAE-Sephacel column chromatography as shown in **Fig. 6** and the active fractions were pooled. All tests of protein kinase with histone as a substrate contained 0.1 mM EGTA. Calcium concentrations are given as free Ca^{2+} in excess of the level of EGTA. **a, b** ●—●, Controls with 0.1 mM EGTA; ■—■, 0.1 mM EGTA and 0.2 mM Ca^{2+}; □—□, 0.1 mM EGTA and 1.1 mM Ca^{2+}. Panel **b** shows the experiment in **a** on a % scale. In panel **c** the dependence of activation of protein kinase on the Ca^{2+} concentration in excess of 0.1 mM EGTA is shown

systems (Gallagher and Leonard 1982; Scherer 1984a).

For tonoplast we used nitrate-inhibited ATPase (Walker and Leigh 1981; Churchill and Sze 1983; Bennett et al. 1984) as a marker which was measured in the presence of 1 mM azide. Azide inhibits mitochondrial ATPase but not tonoplast H^+-ATPase, and both ATPases are inhibited by nitrate so that without azide both ATPases would be measured (Wang and Sze 1985). By comparison of this marker with the profile of ATPase stimulation by the PAF and the partially purified protein kinase (Fig. 9b), it is suggested that tonoplast H^+-ATPase is also stimulated, as was proposed by the proton-pumping experiments with microsomes (Fig. 1–3). However, the contribution of ATP-hydrolysing enzymes other than H^+-ATPase may have obscured the effect of PAF and the partially purified protein kinase on ATP hydrolysis by the tonoplast H^+-ATPase, so that this may be a reason for the partial correlation of marker enzyme and ATPase stimulation. Therefore, it is difficult to exclude contributions of stimulation by the PAF of ATP hydrolysis in other compartments, e.g. Golgi membranes.

Discussion

Our finding that a rare animal phospholipid, platelet-activating factor, stimulates proton transport in isolated plant membrane vesicles was at first peculiar and unexplained (Scherer 1985). One obvious explanation seemed to be that the PAF might act as a detergent on plant membranes, as was assumed by others because of its effect on glucansynthetase II (Kauss and Jeblick 1986). Our earlier results argued against this possibility since the effect of the PAF on proton transport is dependent on the ionic environment. Its effect is optimal in the presence of phosphate as a permeant anion (Scherer 1985; Scherer and Martiny-Baron 1987) and is absent in more than 10^{-5} M Ca^{2+} (Scherer 1985). Since the stimulation of ATP hydrolysis is not dependent on the presence of phosphate (Scherer and Martiny-Baron 1987; see also Fig. 9) this would be difficult to reconcile with a detergent-like effect of the PAF on membranes.

Therefore, we rather thought that the PAF might interact with a plant protein, perhaps with the H^+-ATPases themselves or with a protein associated with them. Another important question was whether the stimulatory action of the PAF was a completely artificial system for plant membranes or whether it mimicked or triggered the action of a natural regulatory system for plant H^+-ATPases. The reconstitution experiment shown in Fig. 5 demonstrated that a soluble protein actually inter-

acted with the PAF and probably catalysed the activation of proton transport (Fig. 6). This experiment provided a first key to our understanding of the effect of the PAF on proton transport in plant membrane vesicles (as shown in Fig. 1 and Table 1).

The second cornerstone was the identification of a phospholipid in plant membranes with chemical and biological properties similar to, if not the same as, those of the PAF (Fig. 1–4; see also Scherer and Martiny-Baron 1987; Scherer and Stoffel 1987). Hence, we postulate that we identified a plant phospholipid, with regulatory activity, which is a cofactor for a soluble protein, likely a protein kinase (Fig. 6–8), and target molecules which may be H^+-ATPases or proteins associated with them in the tonoplast and plasma membrane (Fig. 9). To verify this, further purification and characterization of the components of the presumed reaction chain is needed.

What could be the role of a PAF-like lipid in plants? Certainly, if the PAF and this lipid prove to be chemically similar or identical, the role of this lipid in plants is quite different from the role of PAF in animals. Platelet-activating factor in animals is a hormone-like compound that is biologically active at nanomolar concentrations. It binds to a receptor protein and triggers further reactions not only in platelets but also in other cell types (Fig. 10; see Hanahan, 1986, for a review). The PAF and its plant counterpart are biologically active in plant membrane vesicles at micromolar concentrations (Table 1, Fig. 3; see Scherer and Stoffel 1987). It is a minor phospholipid in plant membranes comprising about 1% of the total phospholipids in zucchini hypocotyl microsomes (Table 2). Hence, we think that this lipid in plants is more likely a membrane constituent and presumably not a hormone or a messenger molecule at the tissue

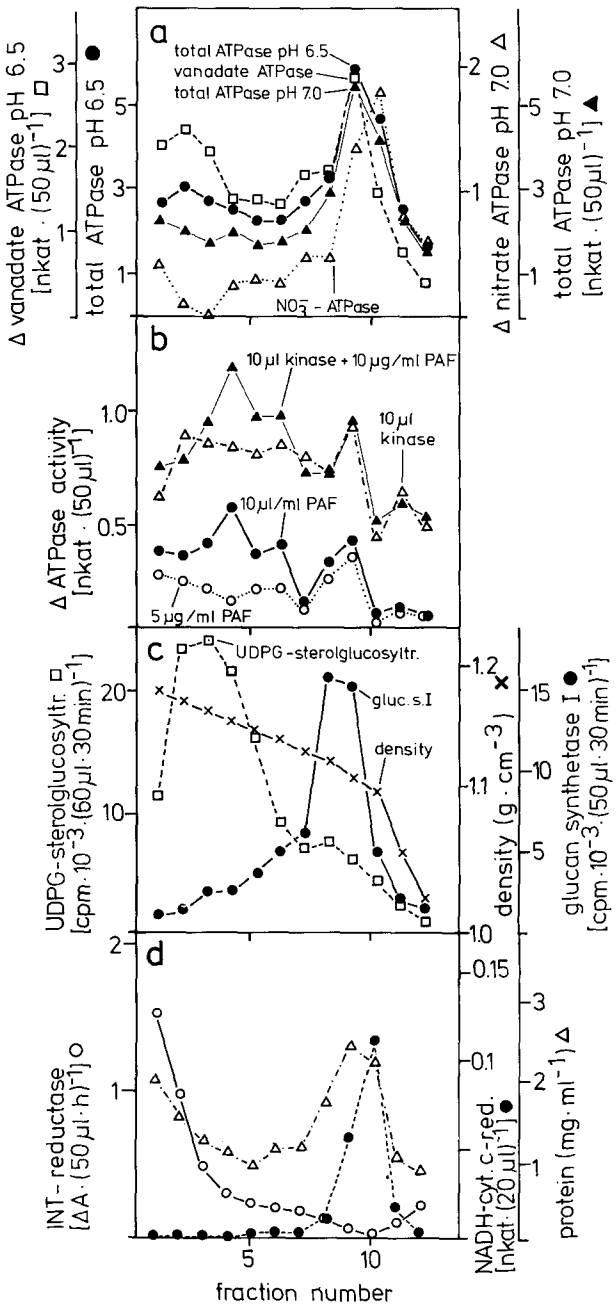

Fig. 9a–d. Stimulation of ATPase activity by the PAF, by partially purified protein kinase, and by both in fractions of a linear sucrose gradient. The linear sucrose gradient of zucchini hypocotyl membranes was prepared as described in *Materials and methods* and centrifuged overnight (16 h). For comparison with other gradients the density of the fractions is indicated in panel **c**. Assays for ATPase were incubated for 20 min and each datum point represents a duplicate assay. All ATPase and marker-activity measurements were made from a single gradient-centrifugation experiment with fractions pooled from six parallel gradients **a** ●—●, Total ATPase activity pH 6.5; ▲—▲, total ATPase activity pH 7.0; □– – –□, difference in ATPase activity at pH 6.5 with and without 0.5 mM sodium orthovanadate in the presence of 1 mM sodium molybdate; Δ– – –Δ, difference in ATPase activity with and without 50 mM nitrate-BTP in the presence of 1 mM sodium azide and 1 mM sodium molybdate. **b** Difference in ATPase activity (pH 6.5) without addition and in the presence of 5 µg·ml^{-1} PAF, (○···○), 10 µg·ml^{-1} PAF (●—●); 10 µl partially purified protein kinase per assay (Δ·–·–·Δ, and 10 µg·ml^{-1} PAF plus 10 µl partially purified protein kinase per assay (▲—▲). **c** □– – –□, UDPG, sterolglucosyltransferase; ●—●, glucan synthetase I (Golgi); ×—×, density. **d** ○—○, *p*-iodonitrotetrazolium violet (*INT*)-reductase; ●– – –●, NADH-cytochrome *c*-reductase; Δ·–Δ, protein

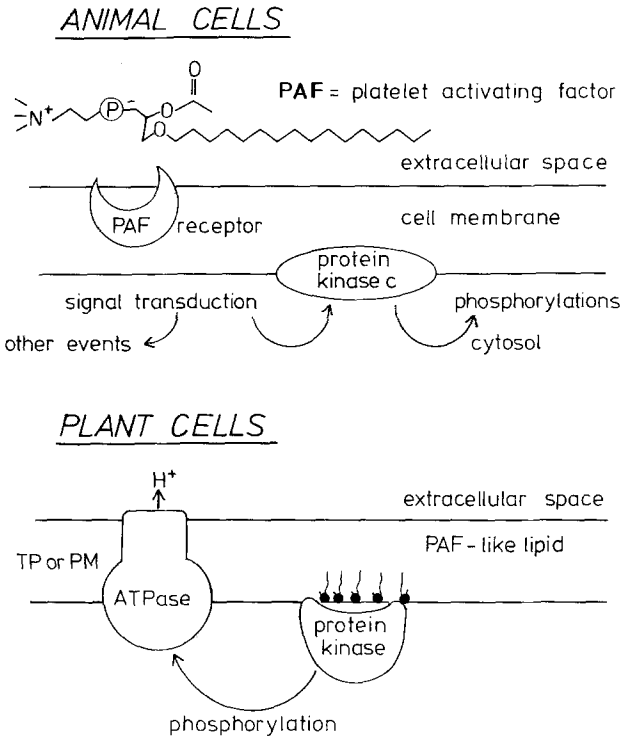

Fig. 10. Mechanism of action of PAF in animal cells (Hanahan 1986) and hypothetical mechanism of action of the PAF-like lipid in plant cells. In animal cells the PAF binds at nanomolar or lower concentrations to a receptor protein in the cell membrane. This triggers Ca^{2+} influx, turnover of phosphorylated phosphatidylinositols, and phosphorylations by protein-kinase C of as yet unidentified proteins. Our results on isolated plant membranes indicate that a plant lipid similar to the PAF is present in plant membranes i.e. presumably also in the tonoplast (*TP*) and the plasma membrane (*PM*). Exogenously added PAF or active plant lipid are thought to stimulate a dissociable protein kinase which could activate H^+-ATPases in tonoplast and plasma membrane by phosphorylation. It is known that plasma-membrane H^+-ATPase consists of a single polypeptide chain of about 100000 Da whereas the tonoplast H^+-ATPase consists of several subunits (Sze 1985). This is not indicated in the schematic drawing and neither are the phosphorylated proteins since they may be only associated with the H^+-ATPases. The effects of the phospholipid-stimulated protein kinase are not implied to be the same for tonoplast and plasma membrane

level. One of our goals is to know the structure of this lipid and its distribution in plant membranes; such information would perhaps give a clue to its functions in plants.

Inasmuch as we have found a phospholipid-(PAF)-dependent protein kinase (Fig. 6–8; see Scherer and Martiny-Baron (1987), this protein kinase could be similar to protein kinase C (Takai et al. 1977; Takai et al. 1979; Kikkawa et al. 1982). Protein kinase C is activated by diglyceride and phosphatidylserine in the presence of 1–5 μM Ca^{2+}, and at unphysiological levels of Ca^{2+} the enzyme is lipid-independent (Takai et al. 1979; Kishimoto et al. 1980; Kaibuchi et al. 1981; Hannun et al. 1986). Phorbol esters can replace diglyceride in the activation of protein kinase C (Nishizuka 1984; Ganong et al. 1986; Hannun and Bell 1986) which eventually leads to tumor growth (Nishizuka 1984). A C-type kinase has been sought in plants as well, and phospholipid-dependent protein kinase has been described for several plants (Schäfer et al. 1985; Olah and Kiss 1986; Elliott and Skinner 1986; Favre and Turian 1987). These plant protein kinases are similar in their chromatographic behavior on DEAE matrices where they elute with 0.2–0.4 M NaCl, as does the protein kinase described here (Fig. 7). Stimulation of protein kinase by Ca^{2+} was also observed in all instances. The plant protein kinases described by other groups were all activated by phosphatidylserine with a variable degree of specificity for this lipid. With wheat an effect of phorbol ester was proposed (Olah and Kiss 1986) but only demonstrated in conjunction with phosphatidylserine, whereas Elliott and Skinner (1986) could not find an effect of phorbol ester. In no instance was an effect of diglyceride alone on protein kinase demonstrated. Phosphatidylserine, diglyceride, and phorbol ester are not active in our system (Table 1). The only remaining similarity of our system to animal protein kinase C is that in the presence of relatively high levels of Ca^{2+} the zucchini enzyme is no longer lipid-stimulated (Fig. 8). This similarity seems too superficial to assume homology between the protein kinase described here and the protein kinase C. A similar conclusion was drawn by others (Elliott and Skinner 1986; Favre and Turian 1987) who found phosphatidylserine to be the only active lipid but not diglyceride or phorbol ester.

The function of protein kinase C is intimately linked to the turnover of phosphorylated phosphatidylinositols with the concommitant generation of two second messengers, diglyceride and inositol-1,4,5-trisphosphate (Berridge and Irvine 1984; Nishizuka 1984). Since we could find no biological activity of diglyceride in our system, an attractive model would be that the phospholipid-dependent and Ca^{2+}-stimulated protein kinase described here is linked to events that trigger the elevation of cytosolic Ca^{2+} in plant cells (Hepler and Wayne 1985; Poovaiah et al. 1987). Inositol-1,4,5,-trisphosphate could be one mediator in the regulation of cytosolic Ca^{2+} in plants (Drøback and Ferguson 1985; Schumaker and Sze 1987) and the protein kinase described here could act in a signal-transmission pathway parallel to a still hypothetical C-like diglyceride-dependent protein kinase in plants (Morré

et al. 1984; Boss and Massel 1985; Strasser et al. 1986; Sandelius and Sommarin 1986; Heim et al. 1987; Morse et al. 1987; Zbell and Walter 1987). A calmodulin-dependent protein kinase that decreased ionophore-stimulated ATPase activity has also been described (Zocchi 1985). Since membrane phosphorylation was conducted in the presence of 0.5 mM Ca^{2+} and of calmodulin whereas our experiments routinely contained 0.1 mM EGTA, both sets of conditions are not comparable and such high Ca^{2+} concentrations would tend to uncouple proton transport via a Ca^{2+}/H^+ antiport (Scherer and Fischer 1985).

Our results can be explained with the assumption that both tonoplast and plasma-membrane H^+-ATPase may be regulated by PAF and a PAF-stimulated protein kinase (Fig. 9), even though the two enzymes have a completely different subunit structure (Sze 1985). Regulation of tonoplast H^+-ATPase has been reported but the mechanism is as yet unexplained (Struve and Lüttge 1987). Phosphorylation by protein kinase of the plasma-membrane H^+-ATPase of yeast (Yanagita et al. 1987) and of higher plants (Bidwai and Takemoto 1987; Schaller and Sussman 1987) has been demonstrated, and increased ATPase activity has been correlated with the phosphorylated state (Portillo and Mazon 1985; Bidwai and Takemoto 1987). Other examples of stimulation of ATPase activity in vitro have been the effect of hormones or fusicoccin (Scherer and Morré 1978b; Scherer 1981, 1984b, c; Gabathuler and Cleland 1985; Rasi-Caldogno et al. 1986). Fusicoccin-dependent phosphorylation of a 33-kDa protein has been reported which indicates a regulatory role for proteins possibly associated with the plasma-membrane H^+-ATPase (Tognoli and Basso 1987). The mechanism of action of all these different effectors might involve, as a common denominator, protein kinases. With the availability of purified components of a possible signal-reaction chain such as the one postulated here, it should become possible to further eludicate the complete function of such regulatory molecules in plant cells.

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