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Plant Defense Response to Fungal Pathogens[1]

Activation of Host-Plasma Membrane H^+-ATPase by Elicitor-Induced Enzyme Dephosphorylation

Rosario Vera-Estrella, Bronwyn J. Barkla, Verna J. Higgins, and Eduardo Blumwald*

Centre for Plant Biotechnology and Department of Botany, University of Toronto, Toronto, Ontario, M5S 3B2 Canada

Elicitor preparations containing the *avr*5 gene products from race 4 of *Cladosporium fulvum* and tomato (*Lycopersicon esculentum* L.) cells near isogenic for the resistance gene *Cf*5 were used to investigate events following the treatment of host plasma membranes with elicitor. A 4-fold increase in H^+-ATPase activity, coincident with the acidification of the extracellular medium, was detected immediately after elicitor treatment. The elicitor-induced stimulation of the plasma membrane H^+-ATPase was inhibited by okadaic acid but not by staurosporine, suggesting that protein dephosphorylation was required for increased H^+-ATPase activity. This observation was confirmed by γ-^{32}P labeling and immunodetection of the plasma membrane H^+-ATPase. Effects of guanidine nucleotide analogs and mastoparan on the ATPase activity suggested the role of GTP-binding proteins in mediating the putative elicitor-receptor binding, resulting in activation of a phosphatase(s), which in turn stimulates the plasma membrane H^+-ATPase by dephosphorylation.

Extensive research in the last few years has disclosed a sequence of biochemical events that appear to participate in activation of plant disease defense reactions. Early in the interaction between the plant host and the pathogen, signals are produced that induce responses in the respective partners. This process involves the interaction between pathogen-associated molecules (elicitors) and putative plant receptors. This recognition is followed by a signal transduction cascade resulting in defense gene activation and the expression of disease resistance in the plant host. Specific recognition between the plant host and the fungal pathogen determines the outcome of the interaction (Keen, 1990). The resistance or susceptibility of host plants to different races of a fungal pathogen is determined by the match of dominant resistance genes in the plant with dominant avirulence genes (*avr* genes) in the pathogen (De Wit, 1992). The *avr* genes, in at least a few well-studied systems, have been shown to code directly or indirectly for elicitor molecules; resistance genes are suggested to code directly or indirectly for receptor molecules (De Wit, 1992).

Emerging evidence indicates that high-affinity receptors for fungal elicitors are located at the plasma membrane of the plant cell (Keen, 1990), and this has focused attention on rapid changes at the cell surface that might be involved in the activation of inducible defense responses. We previously reported that one race-specific elicitor, the putative product of the avirulence gene *avr*5 of the fungus *Cladosporium fulvum*, induced a rapid increase in active oxygen species, peroxidases, and phenolic compounds in tomato (*Lycopersicon esculentum* L.) cells carrying the complementary resistance gene *Cf*5. We proposed that these rapid changes at the cell surface are involved in the activation of inducible defense responses via a high-affinity receptor located in the plasma membrane of the host cell (Vera-Estrella et al., 1992).

Because the plant plasma membrane is expected to be the site where elicitor-receptor interaction will initiate a signal transduction pathway leading to host resistance, we studied the effect of fungal elicitors on the host plasma membrane H^+-ATPase. Here, we demonstrate that isolated and purified plasma membranes of tomato cells exposed to a race-specific elicitor of *C. fulvum* showed an increase in the plasma membrane H^+-ATPase activity, and we give evidence that the stimulation of the H^+-ATPase is a result of the protein being dephosphorylated by a membrane-bound phosphatase possibly activated through a G protein.

MATERIALS AND METHODS

Plant Material

Tomato cell suspensions derived from a line of tomato (*Lycopersicon esculentum* L.) cv Moneymaker near isogenic for the *Cladosporium fulvum* resistance gene *Cf*5 were grown in 500-mL Erlenmeyer flasks containing 100 mL of Murashige and Skoog medium in the dark at 25°C on a rotatory shaker at 120 rpm and subcultured weekly (Vera-Estrella et al., 1992). Cells used for all experiments were 3 to 4 d old.

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* Corresponding author; fax 1–416–978–5878.

Abbreviations: ADP(β)S, adenosine-5′-*O*-(2-thiodiphosphate); ATP(γ)S, adenosine-5′*O*-(3-thiotriphosphate); G protein, GTP-binding protein; GDP(β)S, guanosine-5′*O*-(2-thiodiphosphate); GTP(γ)S, guanosine-5′-*O*-(3-thiotriphosphate); IF, intercellular fluid.

Production of Specific Elicitors

IFs (obtained by infiltration and centrifugation of the infected leaflets) from tomato leaf tissue infected with *C. fulvum* were prepared according to the method of De Wit and Spikman (1982) with the incompatible fungal races 4 and 2.3. and the compatible fungal races 2.4.5., 2.4.5.9., and 5 and cv Bonny Best (no known *Cf* genes). Control IF was obtained from uninoculated plants placed in the same conditions in a separate growth chamber. For partial purification, the IFs were precipitated with acetone (90%), and the pellet was then freeze dried, resuspended in distilled water to give the original volume, and stored at −20°C. No further purification was carried out, and, consequently, preparations produced in this manner for each race will be referred to as race-specific IFs. These preparations would be expected to contain specific elicitors for each of the *Cf* genes on which the specific race is avirulent, i.e. on which a resistant (incompatible) reaction occurs. Concentrations of IFs used in experiments with tomato cell suspensions or purified plasma membranes were based on the minimum dilution of IF that induced necrosis on leaves of line *Cf*9 plants. Generally, a ratio of IF to water of 1:64 was used, and this contained between 1.25 and 1.40 μg of protein μL^{-1}.

Isolation of Plasma Membranes

Plasma membrane-enriched fractions were isolated and purified by homogenization of 4-d-old *Cf*5 tomato cells, followed by Suc gradient centrifugation. The microsomal portion was fractionated through a discontinuous Suc gradient, and the membranes were collected at the 32 to 38% (w/v) gradient interface. Purity of the membranes was tested as described previously (Blumwald and Poole, 1987).

Measurement of ATPase Hydrolytic Activity

Hydrolytic activity of the plasma membrane ATPase was measured by the release of Pi according to the method of Ames (1966). Plasma membrane vesicles (25 μg of protein) were incubated in 1 mL of solution containing 100 μM sodium molybdate, 10 mM sodium azide, 5 μM gramicidin-D, 3 mM Tris/ATP (pH 6.5), 3 mM $MgSO_4$, 50 mM KCl, 30 mM Tris/Mes (pH 6.5). The assay was carried out in the presence or absence of race-specific or control IF (12 μg of protein). The protein concentration of the IF was equivalent to that reported previously to induce a defense response in cells (Vera-Estrella et al., 1992). The reaction medium was incubated at 37°C for 30 min, the reaction was stopped by adding Ames solution (1 volume of 10% ascorbic acid and 6 volumes of 0.42% ammonium molybdate in H_2SO_4), and the A_{820} was measured. The values are presented as μmol of Pi released h^{-1} mg^{-1} of plasma membrane protein.

Coupled Assay for ATPase Activity

ATPase activity was measured as the rate of ADP-dependent NADH oxidation in a coupled system containing NADH, phospho*enol*pyruvate, pyruvate kinase, and lactate dehydrogenase as previously described (Blumwald and Poole, 1985). The rate of ADP formation was measured in a 1-mL reaction volume containing 50 μg of membrane protein, 50 mM KCl, 0.6 mM Tris/ATP, 3 mM $MgSO_4$, 30 mM Tris/Mes (pH 6.5), 3 mM phospho*enol*pyruvate, 0.184 mM NADH, 2 units of lactate dehydrogenase, and 2 units of pyruvate kinase and in the presence or absence of race 4 or control IF (24 μg of protein). The rate of ADP-dependent NADH oxidation was measured as the decrease in A_{340} with time. Inhibitors and activators were added either before or after the addition of membrane protein as indicated in the legends to the figures.

Measurement of Changes in pH

Four-day-old *Cf*5 cell suspensions (20 mL) were centrifuged at 1000*g* for 10 min and resuspended in 0.3 μM Tris/Mes buffer (pH 6.5). Cells (1×10^5 cells mL^{-1}) were treated by the aseptic addition of the incompatible race 4 and 2.3 IFs, control IF, buffered race 4 IF, and IFs from compatible races. The concentration of IF used for the experiments was based on the minimum dilution (1:64) that induced necrosis in leaves of near-isogenic line *Cf*9, the line giving the highest necrotic response (Vera-Estrella et al., 1992). Generally a 1:64 IF dilution contained 1.4 μg μL^{-1} of protein. Immediately following the addition of different IF, the pH of the solution was measured using a pH electrode attached to a digital potentiometer (Fisher, Unionville, Ontario, Canada). Measurements were taken at 10-min intervals for the 1st h and 30-min intervals for the 2nd h, followed by 60-min intervals for the last 2 h.

SDS-PAGE

Membrane proteins were precipitated by diluting the samples 50-fold in 1:1 (v/v) ethanol:acetone and incubating for 3 h at −20°C, followed by centrifugation at 13,000*g* for 20 min at 4°C. Pellets were air dried, resuspended with Laemmli (1970) sample buffer (4% SDS), and heated at 60°C for 2 min before loading onto 10% (w/v) linear acrylamide gels. After electrophoresis, the gels were stained for 30 min with Coomassie brilliant blue R250 (0.25% in 50% methanol/7% acetic acid), destained in 1:1:8 (v/v/v) methanol:acetic acid:distilled water for 24 h, and dried under vacuum at 80°C for 2 h.

Western Immunoblotting

For western blot analysis, SDS-PAGE-separated membrane proteins were electrophoretically transferred onto a nitrocellulose membrane as previously described (Barkla and Blumwald, 1991). Following electrophoresis, membranes were blocked with Tris-buffered saline containing 0.05% Tween-20 and 5% fat-free milk powder (Carnation) for 2 h at room temperature. Membranes were then incubated overnight at room temperature with a primary antibody raised in rabbit against the C-terminal region of the 100-kD plasma membrane H^+-ATPase from *Avena sativa* (1:1000 dilution). Immune complexes were detected using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G secondary antibody (1:7500 dilution) and adding the colorimetric substrate mixture 5-bromo-guanosine 5′-triphosphate-4-chloro-3-indolyl phosphate/nitroblue tetrazolium for approximately 1 min.

Phosphorylation Assays

Plasma membrane protein (80 μg) was incubated in 500 μL of phosphorylation medium containing 10% glycerol, 6 mM Tris/Mes (pH 6.5), 2 mM DTT, 250 mM mannitol, 5 mM $MgSO_4$, 5 mM KCl, and either race 4 or control IF (40 μg of protein). The phosphorylation reaction was initiated by the addition of 10 μCi of [γ-^{32}P]ATP (2 pmol of ATP). Unless specified, the reaction was stopped after 5 min of incubation at 25°C by adding 10% Laemmli sample buffer (Laemmli, 1970) to the reaction mixture. Aliquots of SDS-denatured proteins (10 μg) were resolved by SDS-PAGE on 10% (w/v) linear acrylamide gels, and the phosphoproteins were detected by autoradiography (X-OMAT AR, Eastman Kodak) for 20 h.

Protein Determination

Total protein was measured by the method of Bradford (1976) with BSA as the standard.

Chemicals

Nucleotides (ADP[β]S, ATP[γ]S, GDP[β]S, and GDP[γ]S) were obtained as lithium salts from Boehringer Mannheim (Laval, Québec, Canada). Staurosporine (from *Streptomyces* sp.), mastoparan (from *Vespula lewisii*), and okadaic acid were purchases from Sigma. Lactate dehydrogenase (from hog muscle) and pyruvate kinase (from rabbit muscle) were from Boehringer Mannheim. [γ-^{32}P]ATP was purchased from Amersham International (Oakville, Ontario, Canada).

RESULTS

Effect of the Race-Specific Elicitors on Plasma Membrane ATPase Activity

The plant plasma membrane H^+-ATPase is known to be subject to strict regulation in vivo by factors such as plant growth regulators, light, and toxins produced by pathogens (Sussman and Harper, 1989). The possible involvement of plasma membrane H^+-ATPase in elicitation of defense responses in tomato was studied using a cell line of tomato near isogenic for the resistance gene *Cf5* and race-cultivar-specific elicitors from *C. fulvum*. To determine whether tomato plasma membranes retain the specific response to the elicitor previously observed in whole cells (Vera-Estrella et al., 1992), plasma membrane-enriched fractions isolated from a *Cf5* line of tomato cells were exposed to race-specific elicitors contained in IFs (diluted soluble apoplastic material) from plants infected with one of a number of races of *C. fulvum*. If the race-cultivar specificity was retained, then only the incompatible combinations (race 4 and 2.3 IFs, containing the *avr5* gene product, and the *Cf5* cell line) would be expected to induce changes in the activity of the plasma membrane ATPase. Treatment of *Cf5* plasma membranes with the race 4 and 2.3 IFs induced a rapid 4-fold increase in the H^+-ATPase activity, whereas the other IFs (all obtained using races compatible with *Cf5* plants) did not induce any significant change in the ATPase hydrolytic activity (Figs. 1 and 2, inset).

The increased activity upon treatment with race 4 and 2.3 IFs could be due to an increase in the H^+-ATPase activity and/or the activity of the Ca^{2+}-ATPase, known to be present in the plant plasma membrane (Briskin, 1990). To determine the individual role of the H^+-ATPase enzyme in the elicitor-induced increased ATPase hydrolytic activity, the pH in the extracellular medium of tomato cells was measured upon addition of the elicitor. If the proton pump was responsible for the increased ATPase hydrolytic activity, an acidification in the extracellular medium of *Cf5* cells exposed to race 4 and 2.3 IFs would be expected. As shown in Figure 2, incubation of *Cf5* cells with the race 4 and 2.3 IFs induced an immediate acidification of the extracellular medium, whereas the exposure of *Cf5* cells to any of the other "compatible" race IFs did not induce any measurable changes in the extracellular pH. Thus, the acidification of the extracellular medium was directly coincident with the fast 4-fold increase in plasma membrane ATPase activity observed when plasma membranes were incubated with race 4 IF in vitro (Fig. 2, inset).

Effect of Race-Specific IF, Okadaic Acid, and Staurosporine on the Protein Phosphorylation

Reversible changes in the phosphorylation status of enzymes are known to be of fundamental importance in the reception and transduction of signals (Ranjeva and Boudet, 1987). Phosphorylation and dephosphorylation of proteins by kinases and phosphatases not only allow the regulation of enzyme activities but also serve as a cascade system for signal amplification (Barford, 1991). The possibility that the elicitor-induced stimulation of the H^+-ATPase was a result of changes in the phosphorylation state of the enzyme was tested using okadaic acid, a specific inhibitor of protein phosphatase 1 and 2A (Haystead et al., 1989), and staurosporine, a general inhibitor of protein kinases (Herbert et al., 1990). Okadaic acid almost completely inhibited the increase in H^+-ATPase activity previously observed in the presence of

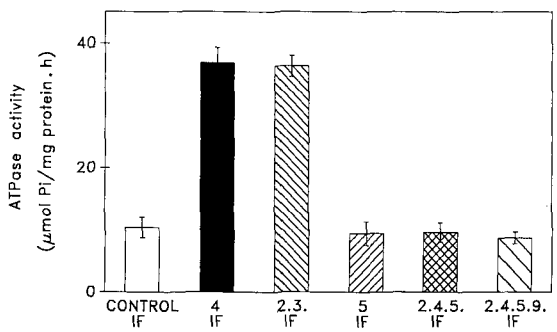

Figure 1. Effect of different IFs, containing specific elicitors of *C. fulvum*, on the ATPase hydrolytic activity of plasma membranes from tomato cell line *Cf5*. The ATPase hydrolytic activity was measured by incubating plasma membrane vesicles (25 μg of protein) in 1 mL of solution containing 100 μM sodium molybdate, 10 mM sodium azide, 5 μM gramicidin-D, 3 mM Tris/ATP (pH 6.5), 3 mM $MgSO_4$, 50 mM KCl, 30 mM Tris/Mes (pH 6.5), and control or race-specific IF (12 μg of protein). IFs of the compatible races 5, 2.4.5, and 2.4.5.9 and incompatible races 4 and 2.3 were used. The values are means ± SD for four experiments with six replicates each.

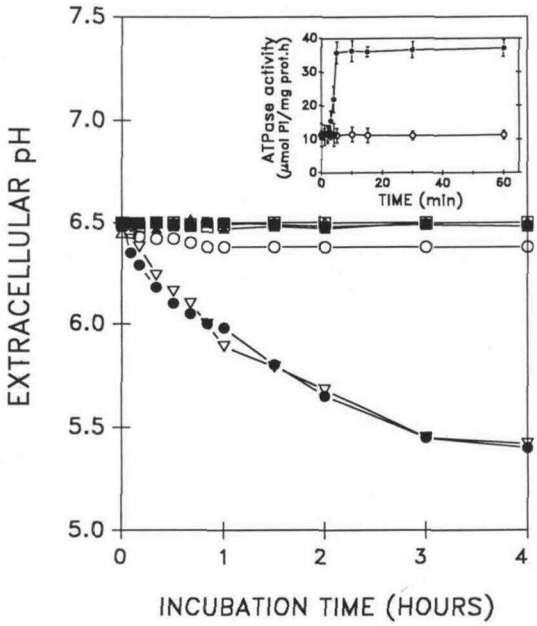

Figure 2. Effect of different IFs, containing elicitor of *C. fulvum*, on the extracellular pH of tomato cell line *Cf5*. Seven-day-old cell suspensions (3×10^5 cells) were centrifuged at 1000*g* for 10 min and resuspended in 3 mL of solution containing 0.3 mM Tris/Mes buffer (pH 6.5) with 3% Suc. IFs from compatible and incompatible fungal races (15 μg of protein) were added, and the pH of the solution was measured. Values are expressed as a means ± SD of four experiments with three replicates each. ●, race 4 IF; ○, control IF; △, control buffer; ▲, race 2.4.5 IF; □, race 2.4.5.9 IF; ■, race 5 IF; ▽, race 2.3 IF. Inset, Time course of the increased plasma membrane ATPase activity of *Cf5* cells by race 4 IF (●) and control IF (○). Values are expressed as a means ± SD of four experiments with three replicates each.

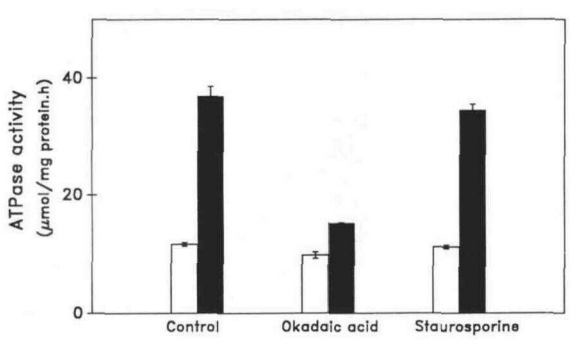

Figure 3. Effect of 0.1 μM okadaic acid and 10 μM staurosporine on the activity of the plasma membrane H^+-ATPase in the presence of control (open bar) or race 4 IF (filled bar). ATPase activity was measured as the rate of ADP-dependent NADH oxidation using 50 μg of membrane protein and 24 μg of control or race 4 IF. Membranes were incubated at room temperature for 5 min with okadaic acid or staurosporine prior to elicitation. IFs were added and the rate of ADP-dependent NADH oxidation was measured as the temporal decrease in A_{340} nm. The values represent the means ± SD of six experiments with three replicates each.

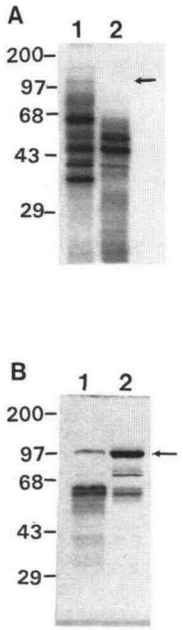

Figure 4. A, Autoradiogram of in vitro [γ-^{32}P]ATP-labeled plasma membrane protein. Labeling was carried out in the presence of control (lane 1) or race 4 IF (lane 2). The arrow represents a polypeptide of 100 kD. B, Western immunoblot of in vitro [γ-^{32}P]-ATP-labeled membrane protein using an antibody against the 100-kD plasma membrane H^+-ATPase from *A. sativa*. Lane 1, Plasma membrane protein incubated for 5 min with control IF. Lane 2, Plasma membrane protein incubated for 5 min with race 4 IF. Photographs are representative of 10 independent experiments.

the race 4 IF (Fig. 3). In contrast, the presence of staurosporine did not affect the increase in ATPase activity induced by the elicitor (Fig. 3). These results indicate that a phosphatase and not a kinase is involved in the elicitor-induced increase in H^+-ATPase activity and that this increased activity is due to dephosphorylation of the enzyme by a phosphatase.

To detect the phosphorylation state of the plasma membrane proteins in the presence or absence of race 4 IF, membranes were labeled with [γ-^{32}P]ATP, and the proteins were then separated by SDS-PAGE. Autoradiograms of plasma membranes exposed to control IF (Fig. 4A) showed a phosphorylated 100-kD polypeptide. In contrast, in the presence of race 4 IF this 100-kD polypeptide appeared to be dephosphorylated (Fig. 4A). The dephosphorylation of other high molecular mass polypeptides and the phosphorylation of some lower molecular mass proteins was also observed (Fig. 4A). Autoradiograms of plasma membranes exposed to compatible IFs (races 5, 2.4.5, and 2.4.5.9) did not show dephosphorylation of the 100-kD polypeptide (results not shown).

Immunodetection of the H^+-ATPase

To determine whether the dephosphorylated 100-kD polypeptide was indeed the 100-kD H^+-ATPase protein, western blot analysis of the γ-^{32}P-labeled plasma membrane protein was carried out using antibodies raised against the C terminus

of the 100-kD H^+-ATPase subunit of *A. sativa*. The antibody strongly cross-reacted with a 100-kD protein in both control and elicitor-treated membrane samples (Fig. 4B), and this polypeptide was coincident with the polypeptide undergoing dephosphorylation in the presence of the race 4 IF (Fig. 4A). Dephosphorylation of the H^+-ATPase was detected 30 s after the addition of race 4 IF (not shown).

The involvement of a phosphatase in the observed elicitor-induced increase of the H^+-ATPase activity was confirmed by γ-^{32}P-labeling of membrane proteins in the presence of okadaic acid (Fig. 5). Although the presence of staurosporine did not affect the dephosphorylation of the 100-kD polypeptide by race 4 IF, the addition of okadaic acid prevented the dephosphorylation.

Effect of Nucleotides and Mastoparan on the Plasma Membrane H^+-ATPase Activity

Elicitor-induced dephosphorylation corresponding to increased H^+-ATPase activity suggested a link between elicitor-receptor recognition and a direct signal transduction pathway. We tested the possible involvement of G proteins in the transduction of a signal initiated by the binding of the elicitor to putative receptors in the tomato plasma membrane by using guanidine nucleotide analogs. GTP(γ)S, a nonhydrolyzable GTP analog that locks G proteins in a GTP-bound active form (Kaziro et al., 1991), induced an increase in ATPase activity comparable to that obtained in the presence of race 4 IF (Fig. 6). GDP(β)S, a nonhydrolyzable GDP analog that locks G proteins in a GDP-bound inactive form (Kaziro et al., 1991), inhibited the increase in ATPase activity induced by race 4 IF (Fig. 6). Incubation of membranes with control nucleotides, ADP(β)S and ATP(γ)S, had no effect on ATPase activity in the presence or absence of race 4 IF (Fig. 6). These results suggest that a G protein might be involved in the activation of protein phosphatases, which in turn dephosphorylate the plasma membrane H^+-ATPase.

The role of G proteins in the transduction of the elicitor/receptor recognition signal was further confirmed using mastoparan, a peptide toxin from wasp venom. This peptide has been shown to promote nucleotide exchange by the G protein through a mechanism similar to that used by native receptors (Higashijima et al., 1990). The addition of mastoparan to the reaction medium containing plasma membrane vesicles induced an increase in the ATPase activity similar to that obtained with GTP(γ)S and with the race 4 IF (Fig 6).

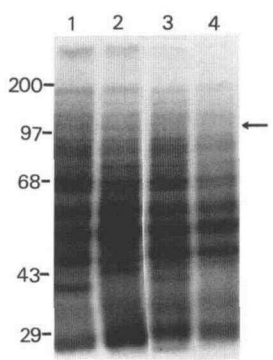

Figure 5. Autoradiogram of in vitro [γ-^{32}P]ATP-labeled plasma membrane protein showing the effect of 0.1 μM okadaic acid and 10 μM staurosporine on the phosphorylation state of the C terminus of the plasma membrane H^+-ATPase. Labeling was carried out in the presence of control/okadaic acid (lane 1), race 4 IF/okadaic acid (lane 2), control/staurosporine (lane 3), and race 4 IF/staurosporine (lane 4). The arrow represents a polypeptide of 100 kD. Photographs are representative of five independent experiments.

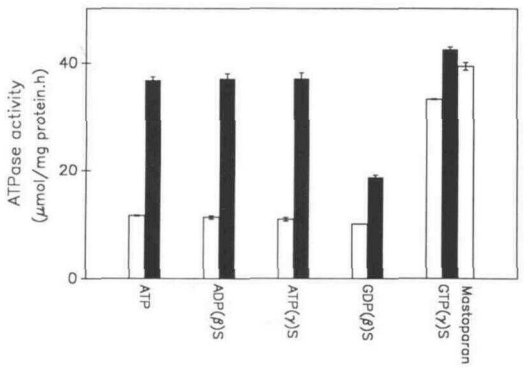

Figure 6. Effect of 100 μM nucleotide analogs or 0.5 μM mastoparan on the ATPase activity of *Cf*5 plasma membranes incubated with either control (open bar) or race 4 IF (filled bar). ATPase activity was measured as the rate of ADP-dependent NADH oxidation in a coupled system. The values represent the means ± SD of six experiments with three replicates each.

DISCUSSION

The interaction between the fungus *C. fulvum* and tomato plant is one of the few host-pathogen systems in which race- and cultivar-specific elicitors have been implicated in determining whether the interaction is compatible or incompatible. The *avr*9 and *avr*4 genes of *C. fulvum* have both been shown to code directly for peptide elicitors that elicit defense responses only on tomato lines with the corresponding *Cf* gene, i.e. the *Cf*9 and *Cf*4 genes, respectively (De Wit, 1992; M. Joosten and P.J.G.M. De Wit, personal communication). To date, knowledge of how these elicitors initially interact with products of the host resistance gene to give specific recognition is unknown; it is assumed that these genes code directly or indirectly for membrane receptors required for the perception of signals by the plant cell and for the initiation of signal generation (De Wit and Flach, 1979). In this study, we used preparations from the apoplast of infected plants (IF) containing the *avr*5 elicitor from race 4 and tomato cells near isogenic for resistance gene *Cf*5 to investigate events following the treatment of the host plasma membrane with the elicitor.

Initially, an array of IF preparations was used to test the effect and the specificity of IFs on the activity of the plasma membrane H^+-ATPase, an enzyme that has been shown to be affected by fungal or bacterial toxins (Briskin, 1990). A rapid 4-fold increase in H^+-ATPase activity, coincident with the acidification of the extracellular medium, was seen only with the incompatible combinations of race 4 IF (*avr*5 elicitor) and *Cf*5 cells (Figs. 1 and 2) or race 2.3 (also containing *avr*5

elicitor) and *Cf*5 cells. These results indicated that purified plasma membranes from *Cf*5 cells retained the specificity previously demonstrated in both intact tomato plants (De Wit and Spikman, 1982) and cell suspensions (Vera-Estrella et al., 1992). Other studies in which tomato cells were used have demonstrated a rapid alkalinization of the extracellular medium in the presence of a yeast extract-derived elicitor, an effect that was blocked by protein kinase inhibitors (Renelt et al., 1993). Atkinson and Baker (1989) also demonstrated an alkalinization of the extracellular medium when tobacco cells were treated with the bacterial pathogen *Pseudomonas syringae*. Likewise, treatment of *Cf*5 cells with a nonspecific elicitor from *C. fulvum* resulted in inhibition of the plasma membrane H^+-ATPase activity, followed by an alkalinization of the extracellular medium (data not shown). Thus, stimulation of the H^+-ATPase and the acidification of the extracellular medium induced by the *avr*5 elicitor suggests that the mode of action of this specific elicitor at the plasma membrane differs from that observed with nonspecific elicitors or pathogenic toxins.

Protein phosphorylation is an effective mechanism for posttranslational modification of proteins. Reversible phosphorylation of proteins by the antagonistic actions of protein kinases and protein phosphatases is considered to be an integral part of basic cellular regulatory mechanisms (Barford, 1991). We investigated the possibility that the increase in H^+-ATPase activity observed in the presence of race 4 IF was due to a change in the phosphorylation state of this enzyme by using inhibitors of protein kinases and phosphatases. The elicitor-mediated stimulation of the H^+-ATPase was significantly reduced by okadaic acid, a protein phosphatase inhibitor, whereas staurosporine, a protein kinase inhibitor, had no effect (Fig. 3). These results suggest that protein dephosphorylation is required for the increased activity of the H^+-ATPase.

The possibility that the ATPase itself is dephosphorylated is supported by in vitro labeling of plasma membranes with [γ-^{32}P]ATP. Immunodetection of [γ-^{32}P]ATP-labeled membranes using an anti-C-terminal H^+-ATPase antibody clearly showed the dephosphorylation of a band coincident with the 100-kD ATPase subunit (Fig. 4) and that labeling in the presence of okadaic acid prevented the elicitor-induced dephosphorylation of this band (Fig. 5). Moreover, our results showed that treatment with the race 4 IF increased the cross-reactivity between the 100-kD polypeptide and the antibody raised against the C terminus of the H^+-ATPase, suggesting that a modification of the H^+-ATPase increased the recognition by the antibody. Changes in membrane protein phosphorylation states by nonspecific elicitors have been reported in soybean (Grab et al., 1989), tomato (Felix et al., 1991), and parsley cells (Renelt et al., 1993). Also, treatment of red beet cells with the bacterial toxin, syringomycin, resulted in phosphorylation of the plasma membrane H^+-ATPase (Suzuki et al., 1992).

It is known that the C terminus of the plasma membrane H^+-ATPase can be reversibly phosphorylated by membrane-bound protein kinase(s) (Serrano, 1989), suggesting that this region of the enzyme may be part of a domain that serves the regulation of the pump activity. In animal cells, phosphorylation of the plasma membrane Na^+,K^+-ATPase resulted in the inhibition of the enzyme activity (Bertorello et al., 1991), and in parsley cells, treatment with an inhibitor of protein kinases resulted in increased plasma membrane H^+-ATPase activity (Kauss et al., 1992). These results suggested activation of the enzyme by the inhibition of its phosphorylation. Our results provide further evidence that dephosphorylation of the plasma membrane H^+-ATPase stimulates the plasma membrane proton pump activity. The signal initiated by elicitor-receptor recognition is likely to be amplified and transduced to effector molecules by signal transduction pathways in the host plasma membrane.

G proteins have been shown to be important components of signal transduction pathways and to transduce extracellular signals into intracellular events through direct coupling to membrane-associated receptors. Activation of these receptors stimulates the binding of GTP to the α subunit, which in turn is released from the heterotrimeric G protein complex. The activated α subunit subsequently modulates its effector, leading to effector activation. In plants, both hormonal (Zaina et al., 1990) and light stimuli (Bossen et al., 1990; Warpeha et al., 1991) are suggested to be transduced through G proteins. GTP and GDP analogs have also been shown to directly regulate both inwardly and outwardly rectifying plasma membrane K^+ channels in mesophyll cells of *Vicia faba* (Fairley-Grenot and Assmann, 1991; Li and Assmann, 1993), and a role for G proteins in mediating a plant defense response has been suggested in the elicitation of the oxidative burst in soybean cell suspensions (Legendre et al., 1992).

The effects of the guanidine nucleotide analogs on the plasma membrane H^+-ATPase activity (Fig. 6), similar to that evoked by race 4 IF, strongly support a role for G proteins in mediating the defense mechanisms in tomato against the fungal pathogen *C. fulvum*. This conclusion is supported by the effects of mastoparan, a 14-residue peptide from wasp venom that has been shown to catalyze G protein activation by mimicking the region on the receptor that regulates the activation of G proteins (Higashijima et al., 1990). The addition of mastoparan also resulted in H^+-ATPase stimulation similar to that observed following elicitor treatment (Fig. 6). The mastoparan-increased H^+-ATPase activity strongly suggests that the *avr*5-specific elicitor may be recognized by cell-surface receptors that in turn activate G proteins. These G proteins could either directly activate protein phosphatases or act indirectly through a secondary messenger system that ultimately activates the plasma membrane H^+-ATPase by dephosphorylation.

In conclusion, our results are consistent with a model by which host plasma membrane receptors bind the fungal elicitor, which triggers G protein activation. This in turn transduces the signal by activating (directly or indirectly) a membrane-bound phosphatase. Activation of the phosphatase results in the stimulation of the plasma membrane H^+-ATPase by dephosphorylation. The increased plasma membrane H^+-ATPase activity and the subsequent acidification of the extracellular medium are important aspects of the initial response of tomato cells to specific elicitors of *C. fulvum*. The increase in H^+ concentration in the cell wall compartment could induce loosening of the cell wall and changes in plasma membrane electrical potential that could alter calcium distribution across the membrane; these events

could trigger the formation of callose (Waldmann et al., 1988), which is deposited in association with conspicuous swelling of mesophyll cells at penetration sites in incompatible, but not compatible, *C. fulvum*-tomato interactions (Lazarovits and Higgins, 1976). *Cf5* tomato cells treated with race 4 IF displayed callose synthesis within a few minutes after elicitor treatment (data not shown). In intact leaves, the synthesis of callose may restrict this fungus to the substomatal space at the infection site, isolating and possibly starving the fungus, which is dependent on host cell nutrients. The subsequent synthesis of phytoalexins (De Wit and Flach, 1979) and hydrolytic enzymes, such as chitinases and β-1,3-glucanases (Joosten and De Wit, 1989), by the resistant plant may further serve to restrict and possibly to digest the fungus.

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