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Regulation of Plant Defense Response to Fungal Pathogens: Two Types of Protein Kinases in the Reversible Phosphorylation of the Host Plasma Membrane H$^+$-ATPase

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The role of reversible phosphorylation of the host plasma membrane H$^+$-ATPase in signal transduction during the incompatible interaction between tomato cells and the fungal pathogen *Cladosporium fulvum* was investigated. Tomato cells (with the *Cf-5* resistance gene) or isolated plasma membranes from *Cf-5* cells treated with elicitor preparations from race 2.3 or 4 of *C. fulvum* (containing the *avr5* gene product) showed a marked dephosphorylation of plasma membrane H$^+$-ATPase. Similar treatment with elicitor preparations from races 5 and 2.4.5.9.11 (lacking the *avr5* gene product) showed no change in dephosphorylation. Elicitor (race 4) treatment of cells, but not of isolated plasma membranes, for 2 hr resulted in rephosphorylation of the ATPase via Ca^{2+}-dependent protein kinases. The initial (first hour) rephosphorylation was enhanced by protein kinase C (PKC) activators and was prevented by PKC inhibitors. Activity of a second kinase appeared after 1 hr and was responsible for the continuing phosphorylation of the H$^+$-ATPase. This latter Ca^{2+}-dependent kinase was inhibited by a calmodulin (CaM) antagonist and by an inhibitor of Ca^{2+}/CaM–dependent protein kinase II. The activation of the Ca^{2+}/CaM-dependent protein kinase depended on the prior activation of the PKC-like kinase.

INTRODUCTION

The resistance of plants to fungal pathogens depends on the ability of the plant to recognize the pathogen early in the infection process. This recognition allows the expression of a series of defense responses, including structural changes in the cell wall that may restrict the pathogen to the site of infection, and the synthesis of hydrolytic enzymes and antifungal compounds that may deter growth of the pathogen (De Wit, 1992).

The recognition of fungal elicitors by host plasma membrane receptors is considered to be among the most important events leading to activation of inducible defense mechanisms (Keen, 1990). Previous studies with tomato cell suspension cultures suggested that the recognition of elicitors from the fungal pathogen *Cladosporium fulvum*, by putative receptors at the host plasma membrane, resulted in the induction of signal transduction pathways that initiated the defense reponses against the pathogen (Vera-Estrella et al., 1994a, 1994b). One of these signals invoked a rapid phosphatase-mediated dephosphorylation of the plasma membrane H$^+$-ATPase, leading to a significant increase in the enzyme activity and subsequent acidification of the extracellular medium (Vera-Estrella et al., 1994a). These results are consistent with a model by which host plasma membrane receptors bind the fungal elicitors and trigger the activation of G proteins (Vera-Estrella et al., 1994a, 1994b). The G proteins then transduce the signal by activating a membrane-bound phosphatase(s), resulting in the dephosphorylation of the host plasma membrane H$^+$-ATPase (Vera-Estrella et al., 1994a), activation of NADH oxidase, and inhibition of ascorbate peroxidase activities (Vera-Estrella et al., 1994b).

Although race-specific elicitor treatments on tomato leaves cause necrosis (De Wit, 1992), comparable treatment of tomato cell suspensions does not result in cell death (Vera-Estrella et al., 1992). In addition, recent histological evidence for a variety of race–cultivar interactions has suggested that host cell death in response to infection by this fungus is not as frequent (Hammond-Kosack and Jones, 1994) as suggested earlier by Lazarovits and Higgins (1976). The plasma membrane H$^+$-ATPase generates the motive force for the transport of nutrients and the regulation of intracellular pH (Serrano, 1989). Prolonged stimulation of the H$^+$-ATPase should result in cell death. The absence of cell death suggests that the initial change in phosphorylation status of the H$^+$-ATPase is further modified to allow treated cells to recover after treatment with the fungal elicitor.

Protein phosphorylation is an effective mechanism for post-translational modification of proteins (Ranjeva and Boudet, 1987; Hubbard and Cohen, 1993). 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). Here, we investigate the reversible phosphorylation of the host plasma membrane H$^+$-ATPase of tomato cell suspensions

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after treatment with race-specific fungal elicitors. Evidence is presented for the involvement of two protein kinases mediating the phosphorylation of the H^+-ATPase after treatment with elicitors, thereby allowing restoration of normal cellular functions.

RESULTS

Effect of Race-Specific Elicitors on the Reversible Phosphorylation of the Host Plasma Membrane H^+-ATPase

The role of reversible phosphorylation in the regulation of enzyme activity and in signal transduction has been discussed previously (Hubbard and Cohen, 1993). Our previous studies have indicated that elicitors from *C. fulvum* induce a phosphatase-mediated dephosphorylation of the plasma membrane H^+-ATPase of *Cf-5* tomato cells in suspension culture, leading to an increase in the enzyme activity. In this study, the possibility of further modifications of the plasma membrane H^+-ATPase following the initial dephosphorylation was studied by examining the in vivo and in vitro phosphorylation/dephosphorylation patterns of the enzyme.

Changes in the phosphorylation status of the plasma membrane H^+-ATPase were monitored in vitro for a period of 2 hr after elicitation with intercellular fluid (IF) from two *C. fulvum* races, race 4 and race 5, incompatible and compatible on plants with the *Cf-5* gene, respectively (Figure 1). The treatment of plasma membranes with race 4 IF resulted in the dephosphorylation of the H^+-ATPase, which was identified as a 100-kD polypeptide by using immunoblotting (Figure 1A). Dephosphorylation was observed for up to 2 hr after treatment. No dephosphorylation of the H^+-ATPase was observed when the membranes were treated for the same periods of time with race 5 IF (Figure 1B). The addition of 0.1 μM $CaCl_2$ in the assays revealed the same pattern (data not shown).

To assess whether race-specific elicitors induced similar effects on the phosphorylation status of the H^+-ATPase in intact cells, the tomato cells were in vivo labeled with phosphorus-32 for 8 hr. After labeling, the cells were treated with race 2.3, race 4, race 5, or race 2.4.5.9.11 IF for periods of time ranging between 0 and 2 hr, and the plasma membranes were isolated immediately after each treatment. Plasma membrane H^+-ATPase was dephosphorylated at 30 min after the treatment with race 2.3 or race 4 IF, but appeared rephosphorylated 1 and 2 hr after the treatment (Figures 2A and 2B). Race 5 or race 2.4.5.9.11 IF did not affect the steady state phosphorylation status of the H^+-ATPase (Figures 2C and 2D). Quantitative changes in plasma membrane H^+-ATPase after each in vivo treatment were examined by immunoblotting, and no significant difference could be detected (Figures 2E to 2H). Increases in the phosphorylation of the H^+-ATPase were inversely correlated with changes in enzyme activity (e.g., 5.7, 12.9, 7.4, and 4.8 μmol of Pi mg protein^{-1} hr^{-1} after treatment with race 4 IF for 0, 0.5, 1, and 2 hr, respectively).

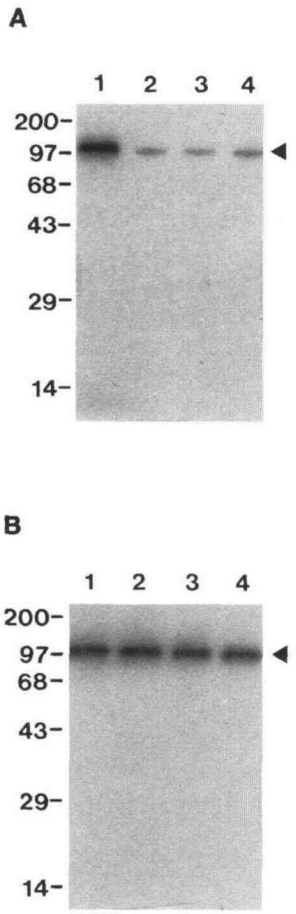

Figure 1. In Vitro γ-^{32}P-ATP–Labeled Plasma Membrane Proteins after Treatment with Elicitor Preparations (IF) from the *C. fulvum* Incompatible Race 4 or Compatible Race 5.

(A) IF from race 4. Cells were grown for 4 days, and plasma membranes were isolated. Plasma membranes were incubated for 15 min in the phosphorylation reaction mixture. Race 4 IF was then added to this mixture (at 0 time). Labeling was stopped by boiling during immunoprecipitation. The immunoprecipitated samples were separated by SDS-PAGE. Lanes 1 to 4 represent in vitro labeling for 0, 0.5, 1, and 2 hr, respectively.

(B) IF from race 5. Experimental procedure and lane settings are as given in **(A)**, except that IF from race 5 (not race 4) was used to treat the plasma membranes.

The arrowheads indicate a 100-kD polypeptide that cross-reacted during immunoblotting with an antibody raised against the 100-kD plasma membrane H^+-ATPase from Arabidopsis. Numbers at left indicate molecular masses of protein standards in kilodaltons. The gels represent seven independent experiments.

Effects of Protein Kinase C Activators and Inhibitors on Phase 1 of the Phosphorylation of the H^+-ATPase in Vivo

Protein phosphorylation has been shown to be a prevalent form of post-translational modification of proteins. Phosphorylation

and dephosphorylation of proteins by kinases and phosphatases are known to be responsible for the regulation of enzyme activities and to serve as a cascade system for the amplification of signals (Ranjeva and Boudet, 1987; Hubbard and Cohen, 1993). Our previous work has suggested that the elicitor-induced dephosphorylation of the host plasma membrane H^+-ATPase is a component of the signal transduction pathway that activates the plant defense response against the pathogen (Vera-Estrella et al., 1994a). To characterize further the mechanisms for rephosphorylation of the H^+-ATPase, two phases of rephosphorylation, that is, phases 1 and 2, were considered. Phase 1 refers to the time interval of 30 to 60 min after the elicitation, and phase 2 refers to the 60 to 120 min after the elicitor treatment. To assess the possible involvement of protein kinase C (PKC), the effects of inhibitors and activators of PKC on the elicitor-induced changes in phosphorylation of the H^+-ATPase during the first hour were studied.

Cf-5 tomato cells were labeled in vivo with phosphorus-32, and the effects of PKC activators on the phosphorylation of the H^+-ATPase were studied by their addition 15 min after the treatment of the cells with race 4 IF. The addition of the PKC activators SC-9, SC-10, and phorbol 12-myristate 13-acetate (PMA) to the cells resulted in the phosphorylation of the H^+-ATPase by 30 min to levels comparable to those observed 1 hr after treatment with the elicitor only (Figure 3, lanes 4 to 6). To corroborate further the role of a PKC-like kinase in mediating the phosphorylation of the H^+-ATPase, four specific PKC inhibitors were added to the cells 30 min after treatment with race 4 IF. Bisindolylmaleimide (20 nM), calphostin C (100 nM), chelerythrine (1 μM), and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004) (50 μM) appeared to inhibit completely the rephosphorylation of the H^+-ATPase when observed 1 hr after the elicitor treatment (Figure 3, lanes 7 to 10). These inhibitor concentrations are either lower than or close to the concentration to inhibit 50% of the PKC activity in animal systems (Asano and Hidaka, 1984; Hidaka et al., 1984; Herbert et al., 1990; Tamaoki et al., 1990; Bruns et al., 1991; Toullec et al., 1991; Barg et al., 1992; Gopalakrishna et al., 1992). The addition of PKC inhibitors to controls did not cause dephosphorylation of the plasma membrane H^+-ATPase, nor

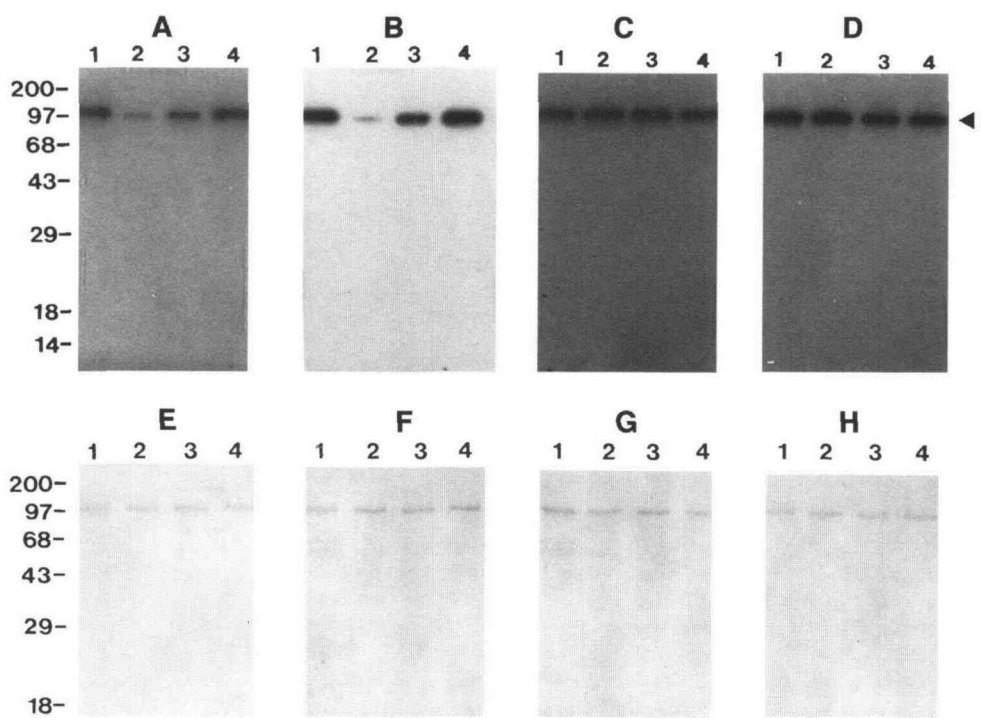

Figure 2. In Vivo ^{32}P-Labeled Plasma Membrane Proteins after Treatment with Elicitor Preparations (IF) from the *C. fulvum* Incompatible Races 2.3 and 4 or Compatible Races 5 and 2.4.5.9.11.

(A) to **(D)** Cells were incubated for 8 hr with carrier-free phosphorus-32, followed by treatment with race 4, race 2.3, race 5, and race 2.4.5.9.11 IFs, respectively, for 0, 0.5, 1, and 2 hr (lanes 1 to 4, respectively). After plasma membrane isolation and immunoprecipitation, the samples were separated by SDS-PAGE. The arrowhead indicates a 100-kD polypeptide that cross-reacted when immunoblotted with an antibody raised against the 100-kD plasma membrane H^+-ATPase from Arabidopsis.

(E) to **(H)** Immunoblot of samples with the antibody corresponding to those in **(A)** to **(D)**, respectively. Lane settings are as given for **(A)**. Numbers at left indicate molecular masses of protein standards in kilodaltons. Gels represent six independent experiments.

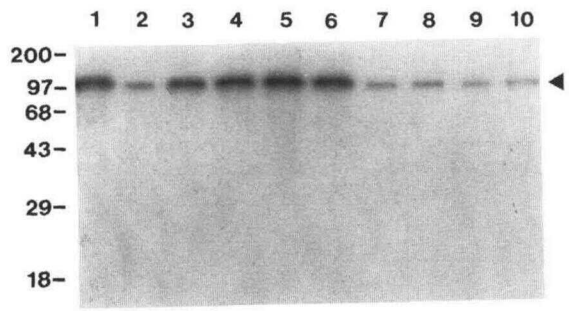

Figure 3. Autoradiography of in Vivo ^{32}P-Labeled Plasma Membrane Proteins Showing the Effect of PKC Activators and Inhibitors on the Phosphorylation Status of the Plasma Membrane H^+-ATPase.

Cells were incubated for 8 hr with carrier-free phosphorus-32, followed by treatment with race 4 IF for 0, 0.5, and 1 hr (lanes 1 to 3, respectively), by treatment with race 4 IF for 0.5 hr but with the addition of 10 μM SC-9, 10 μM SC-10, or 250 nM PMA at 15 min, followed by incubation for another 15 min (lanes 4 to 6, respectively), or by treatment with race 4 IF for 60 min but with the addition of 20 nM bisindolylmaleimide, 100 nM calphostin C, 1 μM chelerythrine, or 50 μM HA1004 at 30 min (lanes 7 to 10, respectively). After plasma membrane isolation and immunoprecipitation, the samples were separated by SDS-PAGE. The arrowhead indicates a 100-kD polypeptide that cross-reacted during immunoblotting with an antibody raised against the 100-kD plasma membrane H^+-ATPase from Arabidopsis. Numbers at left indicate molecular masses of protein standards in kilodaltons. Gels represent six independent experiments.

did these inhibitors and activators cause significant quantitative changes in enzyme levels, as determined by immunoblotting (data not shown).

Effect of Bisindolylmaleimide and Staurosporine on Phase 2 of the Phosphorylation of the Plasma Membrane H^+-ATPase in Vivo

Reversible phosphorylation of proteins is a process involving more than one type of phosphatase or protein kinase (Hubbard and Cohen, 1993). The possible involvement of different protein kinases was studied by applying bisindolylmaleimide (a highly selective inhibitor of PKC) or staurosporine (a more general inhibitor for protein kinases) in the second hour after elicitor treatment. Cells labeled in vivo with phosphorus-32 were treated with race 4 IF, and bisindolylmaleimide or staurosporine was added to the culture medium 60 min later. One hour after the addition of the inhibitors, the cells were disrupted, the plasma membranes were isolated, and the phosphorylation of the H^+-ATPase was compared. Rephosphorylation of H^+-ATPase was completely inhibited by staurosporine but not by bisindolylmaleimide (Figure 4). The two inhibitors caused no significant quantitative changes in plasma memrane H^+-ATPase content, as determined by immunoblotting (data not shown).

Effect of EGTA, W-7, and KN-62 on in Vivo Rephosphorylation of the H^+-ATPase in the Two Phases

The PKC family comprises two groups of enzymes differing in their requirements for Ca^{2+}. One group requires Ca^{2+} for activation, whereas the other lacks the putative Ca^{2+} binding domain and its activity is independent of Ca^{2+} (Nishizuka, 1986). When EGTA, a Ca^{2+} chelator, was added to the cells 30 min after the addition of race 4 IF, the rephosphorylation of the H^+-ATPase (observed 1 hr after elicitor treatment) was inhibited (Figure 5A, lane 3), thus suggesting that the PKC-like kinase mediating the phosphorylation of the enzyme in phase 1 was dependent on Ca^{2+}. Transient changes in cytoplasmic Ca^{2+} concentrations regulate a wide variety of cellular processes. Many of these effects are mediated by calmodulin (CaM) or one of its homologs (Carafoli, 1987). Upon binding with Ca^{2+}, CaM can associate with and modulate the activity of many enzymes, including several serine/threonine protein kinases (Smith et al., 1993). The addition of the CaM antagonist W-7 (Itoh and Hidaka, 1984) 30 min after the cells were treated with race 4 IF did not affect the rephosphorylation of H^+-ATPase (Figure 5A, lane 4), whereas the addition at 30 min of KN-62, a specific inhibitor of Ca^{2+}/CaM–dependent kinase II (CaMKII; Tokumitsu et al., 1990), only slightly affected the rephosphorylation (Figure 5A, lane 5). The addition of EGTA,

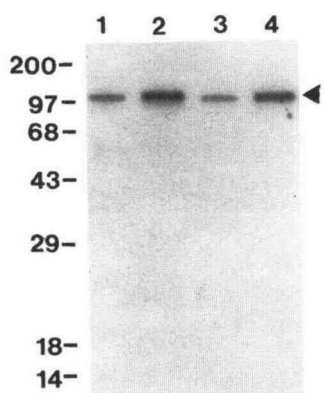

Figure 4. In Vivo ^{32}P-Labeled Plasma Membrane Proteins Showing the Effect of Different Protein Kinase Inhibitors on the Phosphorylation Status of the Plasma Membrane H^+-ATPase.

Cells were incubated for 8 hr with carrier-free phosphorus-32, followed by treatment with race 4 IF for 1 and 2 hr (lanes 1 and 2, respectively) or by treatment with race 4 IF for 2 hr but with the addition of 1 μM staurosporine or 20 nM bisindolylmaleimide at 60 min (lanes 3 and 4, respectively). Following plasma membrane isolation and immunoprecipitation, the samples were separated by SDS-PAGE. The arrowhead indicates a 100-kD polypeptide that cross-reacted during immunoblotting with an antibody raised against the 100-kD plasma membrane H^+-ATPase from Arabidopsis. Numbers at left indicate molecular masses of protein standards in kilodaltons. Gels represent three independent experiments.

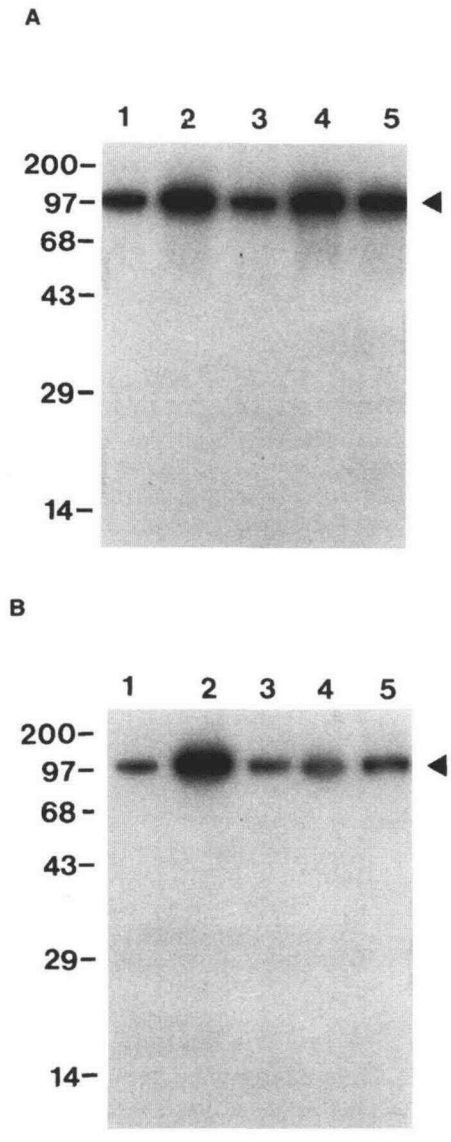

Figure 5. In Vivo ^{32}P-Labeled Plasma Membrane Proteins Showing the Effect of EGTA, W-7, and KN-62 on the Phosphorylation Status of the Plasma Membrane H^+-ATPase.

(A) Cells were incubated for 8 hr with carrier-free phosphorus-32, followed by treatment with race 4 IF for 0.5 and 1 hr (lanes 1 and 2, respectively) or with race 4 IF for 1 hr but with the addition of 1 mM EGTA, 100 μM W-7, or 3 μM KN-62 at 30 min (lanes 3 to 5, respectively). **(B)** Cells were incubated for 8 hr with carrier-free phosphorus-32, followed by treatment with race 4 IF for 1 and 2 hr (lanes 1 and 2, respectively) or with race 4 IF for 2 hr but with the addition of 1 mM EGTA, 100 μM W-7, or 3 μM KN-62 at 1 hr (lanes 3 to 5, respectively). After plasma membrane isolation and immunoprecipitation, the samples were separated by SDS-PAGE. The arrowheads indicate a 100-kD polypeptide that cross-reacted during immunoblotting with an antibody raised against the 100-kD plasma membrane H^+-ATPase from Arabidopsis. Numbers at left indicate molecular masses of protein standards in kilodaltons. Gels represent three independent experiments.

W-7, or KN-62 to the cell culture 1 hr after treatment with race 4 IF prevented the rephosphorylation of the plasma membrane H^+-ATPase previously observed 2 hr after race 4 IF treatment. This indicates that the activity of the second protein kinase was dependent on both Ca^{2+} and CaM (Figure 5B). In animal systems, CaMKII phosphorylates many proteins and is a heteropolymer of subunits (each containing a regulatory and a catalytic domain) that regulates a variety of cellular functions, including carbohydrate metabolism, neurotransmitter synthesis and secretion, cytoskeletal functions, and regulation of ion channels (Hanson and Schulman, 1992). EGTA, W-7, and KN-62 caused no significant quantitative changes in the amount of plasma membrane H^+-ATPase in the designated two phases, as determined by immunoblotting (data not shown).

Effect of Race-Specific Elicitors on PKC-like and Ca^{2+}- and CaM-Dependent Protein Kinase Activities

After treatment with race 4 IF, cells were sampled from 0 to 2 hr and cytosolic extracts were prepared for kinase measurements. The activity of the Ca^{2+}-dependent protein kinase, probably a PKC-like kinase, was shown to peak 40 min after elicitor treatment and declined quickly afterward (Figure 6). The activity could not be detected in the microsomal mem-

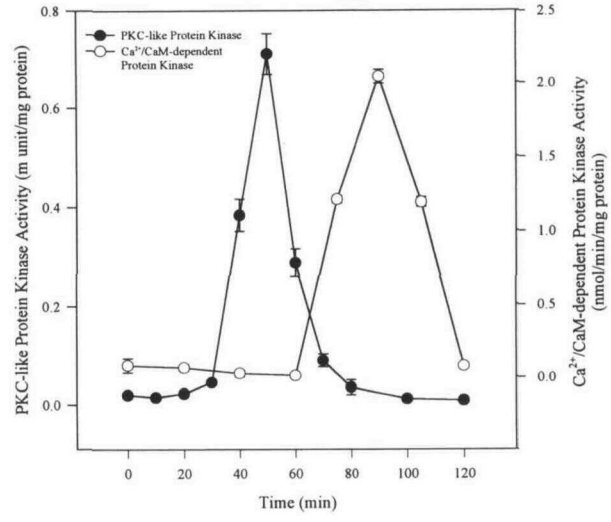

Figure 6. PKC-like Protein Kinase Activity and Ca^{2+}/CaM–Dependent Protein Kinase Activity in Cytosolic Extracts from Cells Treated with Race 4 IF.

Four-day-old cells were treated as described in Methods for in vivo phosphorus-32 labeling, except that phosphorus-32 was not added to the culture medium. Ca^{2+}/CaM–dependent kinase activity is expressed as phosphate transferred per minute per milligram of protein. Data show the mean values (± SE) of three independent experiments. Symbols without error bars indicate that the bars are shorter than the symbols.

brane fractions isolated from the same samples at any time up to 2 hr after race 4 IF treatment. Ca^{2+}/CaM–dependent protein kinase activity was shown to be low in phase 1 (<1 hr) but increased during phase 2 (>1 hr) (Figure 6). No elevated kinase activity of either type was detected in similar extracts from cells treated with race 5 IF (data not shown).

Dependence of Ca^{2+}/CaM-Dependent Kinase Function on PKC-like Kinase Activity

At least two different kinases appear to be involved in the rephosphorylation of the plasma membrane H^+-ATPase. The first appears to be a Ca^{2+}-dependent PKC-like kinase, whereas the second is a Ca^{2+}/CaM–dependent kinase. To study the relationships between the activity of these two types of kinases, bisindolylmaleimide was added 30 min after the treatment with race 4 IF. The rephosphorylation was completely inhibited at both 1 and 2 hr after race 4 IF treatment (Figure 7). Nevertheless, when bisindolylmaleimide was added to the cells 1 hr after treatment with race 4 IF, the rephosphorylation of plasma membrane H^+-ATPase was not inhibited (Figure 4). A further corroboration of the dependence of the Ca^{2+}/CaM–dependent protein kinase on the induction of the PKC-like protein kinase was provided by the effects of inhibitors on kinase activities. The addition of KN-62 at 30 min or 1 hr after treatment of the cells with race 4 IF inhibited only Ca^{2+}/CaM–dependent protein kinase activity, whereas the addition of bisindolylmaleimide 30 min after treatment of the cells with race 4 IF completely inhibited both kinase activities (data not shown). The time-dependent inhibitory effect of bisindolylmaleimide on the phosphorylation catalyzed by Ca^{2+}/CaM–dependent kinase suggests that this kinase requires the PKC-like kinase for its activation.

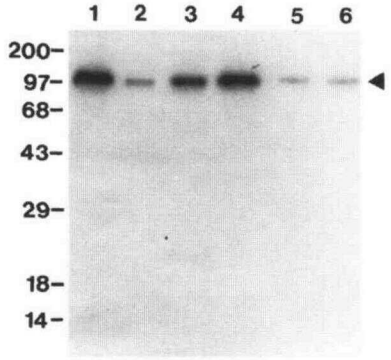

Figure 7. In Vivo ^{32}P-Labeled Plasma Membrane Proteins Showing the Effect of PKC Inhibitor on the Phosphorylation Status of the Plasma Membrane H^+-ATPase.

Cells were incubated for 8 hr with carrier-free phosphorus-32, followed by treatment with race 4 IF for 0, 0.5, 1, and 2 hr (lanes 1 to 4, respectively) or with race 4 IF for 1 and 2 hr but with the addition of 20 nM of bisindolylmaleimide at 30 min (lanes 5 and 6, respectively). Following plasma membrane isolation and immunoprecipitation, the samples were separated by SDS-PAGE. The arrowhead indicates a 100-kD polypeptide that cross-reacted during immunoblotting with an antibody raised against the 100-kD plasma membrane H^+-ATPase from Arabidopsis. Numbers at left indicate molecular masses of protein standards in kilodaltons. Gels represent three independent experiments.

DISCUSSION

The interaction between the leaf mold pathogen *C. fulvum* and tomato has been extensively investigated (De Wit, 1992; Joosten et al., 1994). It is now well documented that plant disease resistance involves a resistance (*R*) gene in the plant that responds specifically to the product of a single avirulence (*avr*) gene in the pathogen (Keen, 1990; De Wit, 1992). The *avr* genes have been shown to encode elicitor molecules either directly or indirectly. These molecules most likely bind to receptors located at the plasma membrane of the host plant cells (De Wit, 1992; Vera-Estrella et al., 1994a). A tomato *Cf-9* gene that may encode a transmembrane receptor has recently been isolated (Jones et al., 1994). Previous work with *Cf-5* cell suspension cultures has shown that treatment with elicitor preparations containing the *avr5* product led to a phosphatase-catalyzed dephosphorylation of the host plasma membrane H^+-ATPase (Vera-Estrella et al., 1994a). Although interveinal injections of such elicitors caused necrosis in tomato leaves, the viability of the cell suspensions did not decline significantly (Vera-Estrella et al., 1992). Likewise, it has recently been suggested that incompatible reactions between tomato and *C. fulvum* rarely initially result in cell death, at least under extreme high humidity (Hammond-Kosack and Jones, 1994). The precisely controlled activity of the plasma membrane H^+-ATPase is of crucial importance for such processes as solute transport and the establishment of a pH gradient across the plasma membrane. Thus, it is reasonable to expect that the initial change in the phosphorylation status of the H^+-ATPase is further modified after the initiation of defense mechanisms so vital processes (dependent on the proton electrochemical gradient generated by the ATPase) are not significantly affected.

Reversible phosphorylation/dephosphorylation has been shown to be one of the mechanisms that cells use to modify the function of the plasma membrane H^+-ATPase (Palmgren, 1991; Sussman, 1992). Analysis of phosphorylation in vivo indicated that at 30 min after treatment of cells there was obvious dephosphorylation of the plasma membrane H^+-ATPase. When the treatment was extended to 1 or 2 hr, the dephosphorylation process was reversed and the rephosphorylation of the enzyme was observed. This rephosphorylation of the H^+-ATPase was not seen in the in vitro labeling experiments using isolated membranes, although the plasma membrane–bound phosphatase activity was enhanced within 10 min after elicitor treatment (Vera-Estrella et al., 1994a) and remained at high levels for 2 hr (data not shown). Thus, our data suggest

that the rephosphorylation of the tomato plasma membrane H^+-ATPase requires a cellular element(s) that is lost during the process of membrane isolation.

PKC is one of the best understood multifunctional serine/threonine kinases in animals, but evidence for PKC in plants is limited (Nishizuka, 1986; Elliott and Kokko, 1987; Nanmori et al., 1994). In this study, three PKC activators (SC-9, SC-10, and PMA) reversed the elicitor-induced dephosphorylation of the enzyme (Figure 3). The addition of the highly selective PKC inhibitors 30 min after the treatment of the tomato cell suspensions with race 4 IF inhibited the rephosphorylation of the ATPase (Figure 3). PKC-like activity was detected in the cytosol of elicitor-treated cells, peaking 45 min after treatment and decreasing to background level after ~60 min (Figure 6).

Ca^{2+}-dependent protein phosphorylation is a major post-translational regulation mechanism involved in signal transduction (Poovaiah and Reddy, 1987; Ranjeva and Boudet, 1987; Roberts and Harmon, 1992). The chelation of Ca^{2+} by EGTA resulted in considerable inhibition of the rephosphorylation of H^+-ATPase (Figure 4). The low levels of phosphorylation still detectable after Ca^{2+} chelation were most probably due to the action of the remaining free cytosolic Ca^{2+}. The requirement of Ca^{2+} in the rephosphorylation of the enzyme could be demonstrated only in intact cells; that is, the addition of Ca^{2+} to isolated plasma membrane did not lead to the rephosphorylation (data not shown). Thus, the inability to detect rephosphorylation of ATPase in vitro was not due to changes in Ca^{2+} concentration during membrane isolation. CaM, usually distributed in both plant and animal cells, is often required for the activity of certain types of protein kinases (Roberts and Harmon, 1992). However, the initial phosphorylation of the H^+-ATPase in vivo was not inhibited by W-7 (a CaM antagonist) if added 30 min after elicitor treatment.

Ca^{2+}-dependent protein kinase (CDPK) appears to be unique to plants and protists and has yet to be demonstrated in animal species (Harper et al., 1993; Roberts, 1993). Although the first (phase 1) kinase identified in our work could be a CDPK, our results with three PKC activators and four highly selective PKC inhibitors strongly suggest that it may represent a PKC-like protein kinase. In addition to CDPK, which can respond to Ca^{2+} without the assistance of CaM, a putative Ca^{2+}/CaM–dependent protein kinase has been cloned from apple (Watillon et al., 1993). This enzyme shares significant sequence identity with rat CaMKII in both the kinase catalytic and CaM binding domains (Watillon et al., 1993). The second (phase 2) kinase identified in our work could represent this type of kinase, because KN-62 (a specific inhibitor of rat CaMKII) and W-7 inhibited the phosphorylation mediated by this kinase when added 1 hr after elicitor treatment.

In conclusion, our results showed that the addition of PKC inhibitors or EGTA 0.5 hr after elicitation reduced the rephosphorylation of H^+-ATPase at 1 hr, whereas the addition of W-7 (CaM antagonist) or KN-62 (CaMKII inhibitor) did not. This period (phase 1) represents the phosphorylation of H^+-ATPase catalyzed by a Ca^{2+}-dependent PKC-like protein kinase. In contrast, the addition of W-7, KN-62, or EGTA 1 hr after elicitation reduced the rephosphorylation of H^+-ATPase at 2 hr, whereas the addition of PKC inhibitors did not. This period (phase 2) represents the phosphorylation of H^+-ATPase catalyzed by a CaMKII-like protein kinase. The inhibition of phosphorylation in phase 1 eliminated the phosphorylation in phase 2 (Figure 7). Thus, the effect of Ca^{2+}/CaM–dependent kinase on the rephosphorylation of plasma membrane H^+-ATPase is conditional and dependent on the transient activation of the PKC-like kinase. This "cross-talk" of more than one protein kinase during the phosphorylation of H^+-ATPase is supported by the fact that the phosphorylation continued for up to 2 hr, whereas the activity of the PKC-like kinase(s) in cytosolic preparations dropped to the background level 1 hr after treatment with the elicitor (Figure 6). This PKC-like kinase may represent a downstream divergence point in different signaling pathways. The conditional regulation has the potential to serve as an integrative mechanism, because two inputs linked to different second messenger systems impinging on the whole network would cause a much greater response than activation of either input alone.

Treatment of the *Cf-5* cells with race 4 IF also resulted in the activation of plasma membrane Ca^{2+} channels (A. Gelli and E. Blumwald, manuscript in preparation) and the inhibition of plasma membrane Ca^{2+}-ATPase (B. Lam and E. Blumwald, manuscript in preparation), elevating the net influx of Ca^{2+} into the cytoplasm. This increase in cytosolic Ca^{2+} concentration would allow the rephosphorylation of plasma membrane H^+-ATPase by Ca^{2+}-dependent protein kinases and the restoration of normal cellular functions. The protein kinases might behave as "negative" elements and would be responsible for ensuring an elicitor-induced response that would be quantitatively appropriate, correctly timed, highly coordinated with other activities of the host plant cells, and/or probably more specifically terminated when the elicitor-induced signal transduction is completed. The identification of the amino acid residues that would be phosphorylated by the two kinases and the correlation of the phosphorylation/dephosphorylation with ATPase function are presently under investigation.

METHODS

Plant Material

Cell suspensions derived from a line of tomato (*Lycopersicon esculentum* cv Moneymaker) with the resistance gene *Cf-5* were grown in 500-mL Erlenmeyer flasks containing 100 mL of MS medium (Murashige and Skoog, 1962). They were grown in the dark at 25°C on a rotatory shaker at 120 rpm and subcultured weekly (Vera-Estrella et al., 1992). Cells suspensions used for all experiments were 3 to 4 days old.

Production of Specific Elicitors

Intercellular fluids (IFs) from tomato leaf tissue infected with *Cladosporium fulvum* were prepared according to the method of De Wit and

Spikman (1982). The *Cf-5* incompatible races 2.3 and 4 or compatible races 5 and 2.4.5.9.11 were inoculated onto the cultivar Bonny Best (no known *Cf* genes). The IFs were precipitated with acetone (90%) and stored at −20°C overnight. The preparations were centrifuged at 1650*g* for 20 min, the supernatant was discarded, and the pellet was resuspended in distilled water and boiled for 30 min followed by centrifugation at 1650*g* for 20 min. (Although boiling results in some loss of activity of the *avr5* elicitors, it effectively inactivates many enzymes in the IF and precipitates a considerable amount of contaminating protein.) The pellet was discarded, and the supernatant was freeze dried. Distilled water was added to give the original volume, and the preparations were stored at −20°C. These preparations contained specific elicitors for each of the *Cf* genes on which the specific race of *C. fulvum* is avirulent (i.e., causes a hypersensitive response). Generally, a ratio of IF to water of 1:32 was used and contained between 0.10 to 0.45 μg of protein per μL. There is substantial evidence demonstrating that unpurified and partially purified IFs exhibit the required specificity for the *Cf* genotypes.

De Wit and Spikman (1982) initially showed that IF from *C. fulvum* races with the *avr5* gene (e.g., races 4 and 2.3) caused necrosis on *Cf-5* plants, whereas IFs from races lacking the *avr5* gene (e.g., races 5 and 2.4.5.9.11) did not cause necrosis. Vera-Estrella et al. (1992), using other indicators of the hypersensitive response (e.g., the oxidative burst, lipid peroxidation, and increased peroxidase activity), showed the same specificity of IFs on cell suspension cultures containing either the *Cf-4* or the *Cf-5* gene. These changes, together with the β-glucanase and chitinase increases reported by Degousse et al. (1994), are all considered to be strongly associated with the plant defense response. The increase in the host plasma membrane H^+-ATPase activity (Vera-Estrella et al., 1994a) and changes in the plasma membrane redox activities (Vera-Estrella et al., 1994b) have also been shown to be specifically induced in *Cf-5* tomato cells by IFs from fungal races with the *avr5* gene.

In Vivo Phosphorus-32 Labeling

Three- to 4-day-old cell suspensions were washed twice with a solution containing 0.3 mM Tris-Mes, pH 5.7, 3% sucrose, and 0.1 μM $CaCl_2$ and were resuspended in a final volume of 80 mL. The cells were then incubated for 8 hr with 1 mCi of carrier-free phosphorus-32. Ten- to 20-mL aliquots were then transferred into small flasks, and the cells were treated with IFs, protein kinase C (PKC) inhibitors, PKC activators, staurosporine, a Ca^{2+} chelator, calmodulin (CaM) antagonist *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), or the Ca^{2+}/CaM–dependent kinase II (CaMKII) inhibitor 1-(*N,O-bis*-[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62) at concentrations and for periods of time indicated in the legends to Figures 3 to 5 and 7. Plasma membranes were then isolated from treated cells and used for immunoprecipitation (described below).

In Vitro Phosphorylation Assays

Plasma membrane protein (described below) was incubated at 25°C for 15 min in phosphorylation buffer containing 10% glycerol, 6 mM Tris-Mes, pH 6.5, 2 mM DTT, 250 mM mannitol, 5 mM $MgSO_4$, 1 μM GTP, 5 mM KCl, and 2 μCi of γ-^{32}P-ATP. IF preparations were added, and after a specified period of incubation at 25°C, the labeling process was stopped by the initiation of immunoprecipitation (the boiling step; see below). Proteins were then resolved by SDS-PAGE on 12.5% (w/v) linear acrylamide gels. The pattern of phosphorylated polypeptides was visualized by autoradiography, using Kodak X-Omat AR autoradiography film.

Plasma Membrane Isolation

Microsomal fractions from *Cf-5* cell suspensions were isolated as described previously (Vera-Estrella et al., 1994a). Microsomes were resuspended in 0.2 mL of phase suspension medium containing 250 mM sucrose, 5 mM potassium phosphate buffer, pH 7.0, and 1 mM DTT. The microsomes were phase partitioned according to Thomson et al. (1993) in an 8- or 4-g phase system with a final composition of 6.5% (w/w) dextran T500, 6.5% (w/w) polyethylene glycol (molecular weight of 3350), 250 mM sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8, and 1 mM DTT. The resulting upper phase was partitioned through two additional steps, and the resulting upper phase from the third partition step was diluted 10-fold in phase-diluting medium containing 250 mM sucrose, 25 mM K_2SO_4, 20 mM Tris-Mes, pH 7.0, and 1 mM DTT. The membranes were collected by centrifugation at 100,000*g* for 40 min at 4°C and resuspended in suspension buffer containing 250 mM sucrose, 10% (v/v) glycerol, 1 mM DTT, and 2 mM Tris-Mes, pH 7.0. The phase-purified plasma membranes were either used immediately or frozen in liquid nitrogen and stored at −70°C until use. For in vivo labeling, all of the above-mentioned buffers were supplemented with 1 μM staurosporine to inhibit kinase activities during the membrane preparation.

Immunoprecipitation

All protein samples used for phosphorylation studies on SDS-PAGE and immunoblotting were obtained with immunoprecipitation by using an antibody raised against the N terminus of the 100-kD plasma membrane H^+-ATPase isolated from *Arabidopsis thaliana*. For immunoprecipitation of the plasma membrane H^+-ATPase, 15 to 25 μg of isolated plasma membrane was boiled for 5 min in 2 × immunoprecipitation buffer (20 mM sodium phosphate, pH 7.2, 300 mM NaCl, 2 mM EDTA, and 3% Triton X-100), followed by centrifugation for 10 min at 15,000*g*. An equal volume of 2 × immunoprecipitation buffer was added to the supernatant together with 5 μL of antibodies. After 10 min of gentle mixing, they were incubated overnight at 4°C. An equal volume of 10% prewashed protein A–agarose was added. (Protein A–agarose was washed with 50 volumes of 1 × immunoprecipitation buffer by mixing and microcentrifugation and resuspended in immunoprecipitation buffer, giving a final concentration of 10%.) The samples were incubated at room temperature for 2 hr before centrifugation for 4 min at 15,000*g* in a microcentrifuge. The supernatant was discarded, and the pellet was washed three times with 1 mL of immunoprecipitation buffer by mixing and microcentrifugation. The pellet was finally resuspended in SDS-PAGE sample buffer (Laemmli, 1970) and centrifuged at 15,000*g*, and the supernatant was used for SDS-PAGE.

SDS-PAGE and Electroblotting of Proteins

Membrane proteins were solubilized as given by Laemmli (1970). Protein separation was conducted by using a discontinuous SDS-PAGE (12.5% separation gel) system. After electrophoresis, the proteins were transferred to nitrocellulose, using a tank blotting chamber.

Immunoblotting

Immunoblotting was performed by using a primary antibody raised in rabbit against the 100-kD plasma membrane H+-ATPase from Arabidopsis.

Ca^{2+}-Dependent PKC-like Kinase Activity

Three- to 4-day-old cell suspensions were treated as described above for the in vivo labeling experiments but without the phosphorus-32. Cytosolic extract was prepared by using the homogenization buffer for microsomal preparation. The homogenate was centrifuged for 60 min at 110,000*g*. The crude extract (<50 mL) was applied to a DE-52 (Sigma) column (8 × 1 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol (buffer A). The column was washed with 50 mL of buffer A and subsequently with 180 mL of buffer A containing 20 mM NaCl. The enzyme was eluted with 50 mL of buffer A containing 90 mM NaCl at a flow rate of 2.5 mL/min. The eluate was concentrated in a concentration cell with a molecular mass cutoff of 55 kD, giving a protein concentration of 2 to 3 mg/mL. The kinase was assayed as described by Kitano et al. (1986), using histone H1 as substrate and γ-^{32}P-ATP as the labeled phosphate donor.

Ca^{2+}/CaM-Dependent Protein Kinase Activity

Cytosolic extract was prepared as described above. The pellet was rehomogenized in 2 volumes of homogenization buffer with a Teflon homogenizer, followed by centrifugation at 110,000*g* for 60 min, and the total supernatant was adjusted to pH 7.5 with HCl. Total activity of Ca^{2+}/CaM-dependent protein kinase was determined according to Tokumitsu et al. (1990), with slight modifications. The assay mixture, containing 35 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.125 μM CaM, was preincubated at 30°C for 2 min. The reaction was initiated by adding 10 μg of synthetic CaMKII substrate, with γ-^{32}P-ATP as the phosphate donor. The reaction was terminated after 5 min, and the incorporation of phosphorus-32 was determined according to Tokumitsu et al. (1990).

Protein Determination

Protein was measured as given by Bradford (1976), using BSA as a standard.

Chemicals

Bisindolylmaleimide, calphostin C, chelerythrine chloride, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004), KN-62, phorbol 12-myristate 13-acetate (PMA), staurosporine, and CaMKII substrates were obtained from CalBiochem (San Diego, CA). W-7, *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9), and *N*-heptyl-5-chloro-1-naphthalenesulfonamide (SC-10) were purchased from Sigma. γ-^{32}P-ATP and phosphorus-32 were purchased from Amersham International. Other chemicals were purchased from either ICN Biomedical (Mississauga, Ontario, Canada) or BDH Inc. (Toronto, Ontario, Canada).

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