The Plant Cell, Vol. 9, 653–664, April 1997 © 1997 American Society of Plant Physiologists

A Functional Homolog of Mammalian Protein Kinase C Participates in the Elicitor-Induced Defense Response in Potato

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The elicitor-induced activation of the potato pathogenesis-related gene *PR-10a* is positively controlled by a protein kinase(s) that affects the binding of the nuclear factors PBF-1 (for *PR-10a* binding factor-1) and PBF-2 to an elicitor response element (ERE). In this study, we have identified a kinase that has properties similar to the conventional isoenzymes of the mammalian protein kinase C (PKC) family. The treatment of potato tuber discs with specific inhibitors of PKC abolished the elicitor-induced binding of the nuclear factor PBF-2 to the ERE. This correlated with a reduction in the accumulation of the PR-10a protein. In contrast, treatment of the tuber discs with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an activator of PKC, led to an increase in binding of PBF-2 to the ERE and the corresponding increase in the level of the PR-10a protein, mimicking the effect seen with the elicitor arachidonic acid. Biochemical characterization of proteins extracted from the particulate fraction of potato tubers demonstrated that a kinase belonging to the conventional isoforms of PKC is present. This was confirmed by immunoprecipitation with antibodies specific to the conventional isoforms of human PKC and in-gel kinase assays. The ability of the immunoprecipitates to phosphorylate the α-peptide (a PKC specific substrate) in the presence of the coactivators calcium, phosphatidylserine, and TPA strongly suggested that the immunoprecipitated kinase is similar to the kinase characterized biochemically. Finally, the similar effects of the various modulators of PKC activity on the elicitor-induced resistance against a compatible race of *Phytophthora infestans* implicate this kinase in the overall defense response in potato.

INTRODUCTION

Several aspects of plant growth and development, including cell division, elongation, and differentiation, can be affected by internal and external stimuli such as hormones, light, and pathogen attacks. In most of these cases, extensive modification of proteins by phosphorylation has been observed (reviewed in Dixon et al., 1994; Ecker, 1995), suggesting that these stimuli are propagated by protein phosphorylation cascades, which are among the most widely used mechanisms for signal transduction in eukaryotic cells (Hubbard and Cohen, 1993). Phosphorylation cascades are known to regulate the activity of many important proteins and enzymes, and the family of protein kinases involved in these reactions is large and diverse (Karin, 1994).

The finding of second messenger–regulated plant protein kinase activities (Trewavas and Gilroy, 1991) strongly reinforced the thinking that kinases may be part of the signaling cascades that regulate many aspects of plant development and physiology. An example of a signal propagated through the action of protein kinases is the plant response to ethylene gas. In mutants of Arabidopsis insensitive to ethylene, the alleles of *ETR1* and *EIN1* appear to function early in the pathway of ethylene perception and therefore are postulated to act as receptors. The deduced amino acid sequence of *ETR1* reveals similarity to prokaryotic two-component signal transduction systems and contains elements necessary for histidine kinase activity. In contrast, mutants that lead to constitutive activation of ethylene-regulated pathways have also been isolated in Arabidopsis. Analysis of the constitutive triple response mutants suggests that the *CTR1* gene is epistatic to *ETR1* and that its deduced amino acid sequence shows similarity to the Raf family of serine/threonine protein kinases (reviewed in Ecker, 1995).

Phosphorylation events in response to pathogen attacks also seem to be integral to signal transduction cascades. For example, when exposed to various elicitors, rapid changes in the profile of protein phosphorylation are observed (reviewed in Dixon et al., 1994). These changes can be blocked successfully with the use of protein kinase inhibitors (Grosskopf et al., 1990; Felix et al., 1991). Components involved in the elicitor-mediated protein phosphorylation are gradually being elucidated. Recently, Suzuki and Shinshi (1995) demonstrated that in tobacco cells, a kinase similar to the mitogen-activated protein (MAP) kinase is transiently activated in response to elicitors. A direct role for a kinase in the plant defense response has been shown with the isolation

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of *PTO* from tomato. This gene encodes a serine/threonine kinase and confers resistance to bacterial speck disease (Martin et al., 1993). Evidence for a phosphorylation cascade in this system during the defense response is emerging with the identification of *Pti1*, which also encodes a serine/threonine kinase and appears to function downstream of Pto (Zhou et al., 1995). Another resistance gene, *Xa21*, which also encodes a protein kinase, has been isolated from rice. This kinase shows similarity to a family of animal receptor kinases and confers resistance to *Xanthomonas oryzae* (Song et al., 1995). However, unlike Pto, components interacting with Xa21 have yet to be identified.

We have previously shown that elicitor treatment of potato tubers leads to an increase in binding of the nuclear factors PBF-1 and PBF-2 to a DNA element important for the activation of the potato pathogenesis-related *PR-10a* gene (Després et al., 1995). The binding of these factors to the elicitor response element (ERE) correlates with the activation of the *PR-10a* gene, which is expressed during the defense response to pathogen attack in potato (Constabel and Brisson, 1992). Treatment of nuclear extracts with alkaline phosphatase abolishes this increased binding, suggesting that a phosphorylation step is involved in this process (Després et al., 1995). The treatment of potato tubers with staurosporine, a potent but nonspecific inhibitor of protein kinases, reduces the elicitor-induced binding of these factors and the accumulation of PR-10a (Després et al., 1995), confirming the involvement of protein kinases in the pathway leading to the activation of *PR-10a*. We now present immunological data and results from enzyme assays that demonstrate the presence and involvement of a homolog of the mammalian protein kinase C (PKC) in the elicitor-mediated defense response in potato. The role of this kinase in the accumulation of the PR-10a protein and in the resistance to *Phytophthora infestans* is further demonstrated by the use of both an activator and inhibitors of PKC.

RESULTS

Inhibitors of Mammalian PKC Affect Elicitor-Induced Expression of *PR-10a*

As our earlier work demonstrated, staurosporine, a protein kinase inhibitor, has major deleterious effects on the elicitor-induced binding of nuclear factors to an elicitor response domain and on the accumulation of the PR-10a protein (Després et al., 1995). Furthermore, the low concentration of staurosporine (5 nM) required to inhibit PR-10a protein accumulation led us to speculate that a kinase belonging to the PKC family is involved in the activation of *PR-10a*. Based on the diverse properties of PKC isoenzymes, Blobe et al. (1995) proposed a strategy to establish a role for PKC in a biological process. One important element of this strategy is to show that inhibitors of this kinase can block the biological event under study. In view of this, we used specific inhibitors of PKC to observe their effects on the elicitor-induced expression of *PR-10a*.

As seen in the immunoblot in Figure 1A, the application of the elicitor arachidonic acid to the tuber discs induced the accumulation of PR-10a (lane 6), compared with the sliced tuber alone (lane 2). The elicitor-induced accumulation of PR-10a protein was severely curtailed when the tuber discs were pretreated with the PKC inhibitors sphingosine (Hannun et al., 1991; Figure 1A, lane 4), bisindolylmaleimide (Toullec

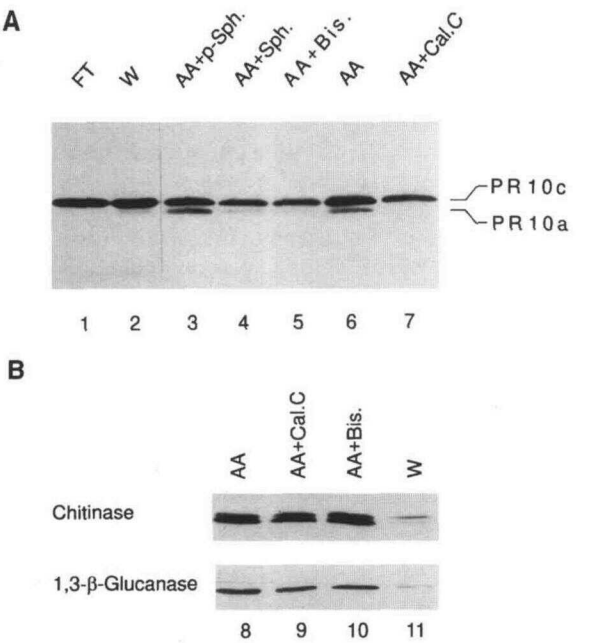

Figure 1. Immunoblot Showing the Effect of PKC Inhibitors on the Accumulation of PR-10a, Chitinase, and 1,3-β-Glucanase.

After a 6-hr aging period, tuber discs were treated with 1% DMSO (lanes 2, 6, 8, and 11), 2 μM palmitoyl sphingosine (AA + p-Sph.; lane 3), 2 μM sphingosine (AA + Sph.; lane 4), 50 nM bisindolylmaleimide (AA + Bis.; lanes 5 and 10), and 500 nM calphostin C (AA + Cal.C; lanes 7 and 9). After 30 min, the elicitor, 1 mg/mL arachidonic acid (AA), was applied to the treated tubers (lanes 3 to 10), and incubation was continued for 48 hr in the dark at room temperature. Total proteins were extracted, and 10-μg aliquots from each sample were separated by SDS-PAGE. Lane 1 represents fresh tuber (FT) tissues, lanes 2 and 11 represent wounded tuber (W) tissues not treated with arachidonic acid, and lanes 6 and 8 represent tuber tissues treated with arachidonic acid.

(A) Immunoblot with anti–PR-10a antibodies. PR-10a and PR-10c indicate the two proteins detected. Essentially identical results were obtained in five independent experiments.

(B) Immunoblot with chitinase and 1,3-β-glucanase as indicated. Essentially identical results were obtained in three independent experiments.

et al., 1991; Figure 1A, lane 5), or calphostin C (Tamaoki, 1991; Figure 1A, lane 7) before the elicitor arachidonic acid was applied on the discs. The fact that palmitoyl sphingosine, an analog of sphingosine that does not inhibit PKC (Hannun et al., 1991), had no effect on the elicitor-induced accumulation of PR-10a (Figure 1A, lane 3) emphasized the specificity of action by sphingosine. The anti–PR-10a antibodies had previously been shown to react against PR-10c, which is encoded by a member of the *PR-10* gene family sharing 70% identity with *PR-10a* (R. Subramaniam and N. Brisson, manuscript in preparation). Figure 1 also indicates that the level of accumulation of PR-10c, which is constitutively present in fresh tubers (Figure 1A, lane 1), was only moderately affected by these inhibitors. Neverthless, nonspecific effects of the inhibitors were monitored with other elicitor-inducible genes. As Figure 1B shows, both chitinase and 1,3-β-glucanase were induced by elicitor treatment (lane 8) compared with the sliced tuber discs (lane 11). However, pretreatment of the tuber discs with the PKC inhibitor calphostin C (Figure 1B, lane 9) or bisindolylmaleimide (lane 10) did not affect the elicitor inducibility of these genes.

An Activator of Mammalian PKC Regulates Expression of *PR-10a*

Other elements of the strategy proposed by Blobe et al. (1995) require that the biological event be mimicked by the addition of PKC activators. Furthermore, this activation should be countered by PKC inhibitors. Studies with animal cells have established that PKC isoforms are potently activated by diacylglycerol (DAG), a product of lipid hydrolysis (reviewed in Nishizuka, 1992). Phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), fully substitute for DAG and are able to activate PKC (Castagna et al., 1982). In contrast to DAGs, phorbol esters are slowly metabolized in the cells, resulting in a protracted stimulation of the PKC enzyme (Blobe et al., 1994). If PKC is involved in the elicitor-mediated response, as implied by the results shown in Figure 1, then application directly on potato tuber discs of phorbol esters such as TPA should lead to the activation of this kinase and result in the activation of *PR-10a.* As the immunoblot in Figure 2 illustrates, treatment of tuber discs with TPA strongly induced the accumulation of the PR-10a protein compared with the wounded tubers (lane W). The inability of 4α-12-*O*-tetradecanoylphorbol 13-acetate (4αTPA), an inactive isomer of TPA, to induce accumulation of PR-10a confirmed the specificity of action by TPA.

The PKC inhibitor sphingosine, which inhibited elicitor-induced accumulation of PR-10a protein (Figure 1, lane 4), is known to be an antagonist of DAGs and phorbol esters (Kahn et al., 1991). Therefore, its ability to counteract the effects of TPA (Figure 2, lane TPA+Sph.) satisfies the other criterion by which the action of an activator is overcome by one of its inhibitors.

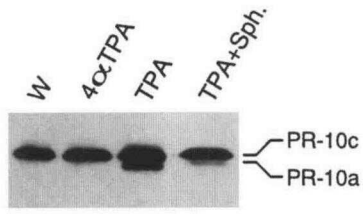

Figure 2. Immunoblot Showing the Effect of TPA on the Accumulation of PR-10a.

After a 6-hr aging period, tuber discs were treated with 1% DMSO (W), 100 nM TPA, 100 nM 4αTPA, and 100 nM TPA plus 2 μM sphingosine (TPA+Sph.). After an incubation period of 48 hr in the dark at room temperature, total proteins were extracted, and 10-μg aliquots of each sample were fractionated by SDS-PAGE and immunoblotted with anti–PR-10a antibodies. PR-10a and PR-10c indicate the two proteins detected. Essentially identical results were obtained in five independent experiments.

Binding of Nuclear Factor PBF-2 Is Affected by TPA and PKC Inhibitors

We demonstrated previously that after elicitor treatment of potato tubers, increased binding of the nuclear factors PBF-1 and PBF-2 to the ERE of *PR-10a* is preceded by a phosphorylation event (Després et al., 1995). This increase in binding of the factors correlates with the activation of *PR-10a.* Now, we have shown that inhibitors of PKC and its activator, TPA, have significant effects on PR-10a protein accumulation (Figures 1 and 2). To confirm that this kinase is indeed involved in the pathway leading to the activation of *PR-10a*, we determined the ability of PBF-2 to bind to the ERE of *PR-10a* in response to the various modulators of PKC activity.

As the results of the electrophoretic mobility shift assay (EMSA) in Figure 3 indicate, elicitor treatment (lane 3) led to an increase in the binding of the factor to the ERE when compared with the control (lane 1). A similar increase in binding was also seen with the extracts from discs treated with TPA, the PKC activator (Figure 3, lane 2). Pretreatment of the discs with the PKC inhibitors calphostin C (Figure 3, lane 4) or staurosporine (lane 5) before application of the elicitor resulted in a level of binding similar to that observed with the untreated discs (lane 1). The notion that phosphorylation mediates this increased binding was reinforced by the observation that alkaline phosphatase treatment (Figure 3, lanes 6 to 10) of the nuclear extracts from both the elicitor and the TPA-treated tuber discs before the assays led to a decrease in the binding of the factor to the ERE (Figure 3, lanes 7 and 8). Binding of the factor was not greatly affected after alkaline phosphatase treatments of the extracts prepared either from combined treatments of elicitor and calphostin C (Figure 3, lane 9), elicitor and staurosporine (lane 10), or from the control tubers (lane 6). These results confirm our previous findings (Després et al., 1995) that indicated that capacity to bind to the ERE of *PR-10a* is

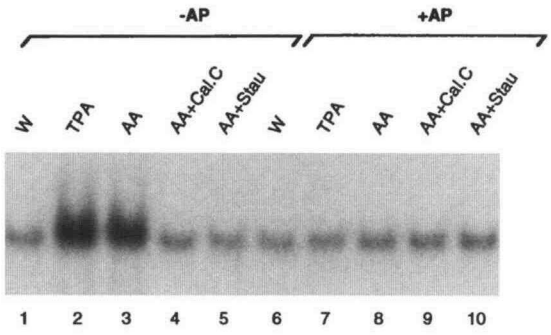

Figure 3. Binding of PBF-2 to a *PR-10a* Promoter Element Is Affected by the Activation of a PKC-like Enzyme and by Treatment with Alkaline Phosphatase.

Tuber discs were aged for 6 hr and treated with 1% DMSO (lanes 1, 3, 6, and 8), 100 nM TPA (lanes 2 and 7), arachidonic acid (AA; lanes 3 and 8), a combination of 500 nM calphostin C and arachidonic acid (AA+Cal.C; lanes 4 and 9), and a combination of 20 nM staurosporine and arachidonic acid (AA+Stau; lanes 5 and 10). The treated discs were incubated for 3 hr in the dark. Nuclear proteins were extracted, and EMSAs were performed with (lanes 6 to 10) or without (lanes 1 to 5) 20 units of alkaline phosphatase (+AP and −AP, respectively). Lanes 1 and 6 represent wounded tuber (W) tissues. The probe is the −135 to −105 DNA fragment that mediates the elicitor induction of *PR-10a* (ERE) (Després et al., 1995). This is representative of at least three different experiments.

potentiated by a phosphorylation step affecting the binding activity of the nuclear factors and places the PKC-like kinase directly in the pathway leading to *PR-10a* expression.

A Homolog of the Conventional Isoforms of PKC Is Present in Fresh Potato Tubers

PKC in animal cells exists as a family of closely related isoenzymes with differences in tissue distribution and intracellular localization (reviewed in Nishizuka, 1995). Currently, the mammalian PKC family consists of 12 different isoenzymes divided into three groups based on biochemical properties and sequence analysis. The first group corresponds to the calcium-dependent conventional isoenzymes α, βI, βII, and γ. These isoenzymes are activated by calcium, phosphatidylserine (PS), and DAGs or phorbol esters. The isoenzymes in the second group, designated the novel group, also require PS and DAGs or phorbol esters but do not require calcium for activation. The third group is made up of the atypical isoenzymes, which are dependent on PS but do not respond to either calcium or DAGs and phorbol esters (reviewed in Hug and Sarre, 1993).

We used proteins isolated from the particulate fraction of fresh tubers to demonstrate the activity of PKC in potato. We used this fraction because in animal cells, PKC translocates from the cytosol to the membrane upon activation (Dutil et al., 1994). This translocation can be induced artificially by isolating the membranes at 15°C with the inclusion of calcium in the homogenization buffer, thereby forcing the kinase into the membranes, where it can be extracted (Hannun et al., 1985). The activity of the kinase was measured by the ability of enzymes present in the particulate fraction to phosphorylate the exogenous substrates histone H1 and α-peptide (a substrate specific for PKC), with the addition of appropriate cofactors (Kitano et al., 1986). The assay mixture was subjected to SDS-PAGE, and the extent of phosphorylation of the substrates was determined by autoradiography (Figure 4). For quantification purposes, some of the experiments presented in Figure 4 were performed three times, and the

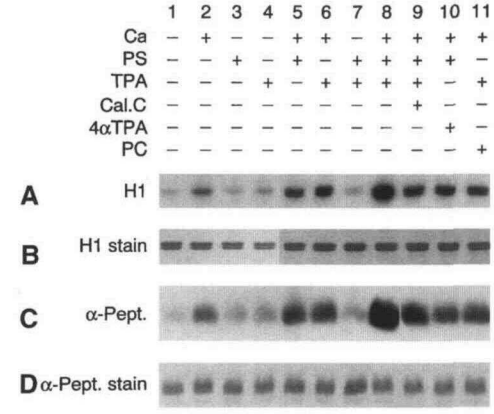

Figure 4. Demonstration of PKC Activity in Protein Extracts of Potato.

PKC activity was measured in the particulate fraction of potato tubers containing the indicated combination of the following compounds: 4.95 mM $CaCl_2$ (Ca), 20 μg/mL PS, 10 nM TPA, 10 nM 4αTPA, 20 μg/mL PC, and 10 μM γ-^{32}P-ATP (1.0 μCi per assay). The assay contained 5 μg of protein. The PKC inhibitor calphostin C (Cal.C) at the concentration of 1 μM was added to an aliquot (100 μg) of the particulate fraction 1 hr before the start of the assay. The final concentration of DMSO was 0.002% in all the reaction mixtures. This is representative of at least four independent experiments. (+) indicates that a compound has been added to the reaction mixture. (−) indicates that a compound has not been added.

(A) PKC activity was measured with histone H1 (200 μg/mL) as substrate. An aliquot (15 μL) of each reaction mixture was separated by SDS-PAGE and visualized by autoradiography.

(B) An aliquot (15 μL) from the reaction mixture in **(A)** was separated as given in **(A)** and visualized by Coomassie blue staining to show relative loading (H1 stain).

(C) PKC activity was measured with the PKC-specific substrate α-peptide (α-Pept.; 10 μM). An aliquot (15 μL) of each reaction mixture was separated by SDS-PAGE and visualized by autoradiography.

(D) An aliquot (15 μL) from the reaction mixture in **(C)** was separated as given in **(C)** and visualized by Coomassie blue staining to show relative loading (α-Pept. stain).

Table 1. Specific Activity of Protein Kinase C in the Particulate Extracts of Potato Tubers

		Histone		α-Peptide	
Lane[a]	Cofactors	Specific Activity[b] (pmol min^{-1} mg^{-1})	Fold Increase	Specific Activity[b] (pmol min^{-1} mg^{-1})	Fold Increase
1	None[c]	164 ± 14.4	1.0	326 ± 39.0	1.0
2	Ca^{2+}	253 ± 13.9	1.5	433 ± 89.0	1.3
5	Ca^{2+} and PS	350 ± 28.0	2.1	977 ± 64.0	3.0
8	Ca^{2+}, PS, and TPA	1630 ± 122.0	9.9	2850 ± 2.0	8.7
9	Ca^{2+}, PS, TPA, and Cal. C[d]	414 ± 87.0	2.5	1180 ± 73.0	3.6
10	Ca^{2+}, PS, and 4αTPA	570 ± 55.0	3.5	1060 ± 190.0	3.2
11	Ca^{2+}, PC, and TPA	414 ± 37.1	2.5	1000 ± 25.0	3.1

[a] Refers to the lanes from Figures 4A and 4C.

[b] Data are expressed as means ± SE ($n = 3$). An aliquot of each assay was analyzed by SDS-PAGE as described in the legend to Figure 4. The substrates were identified by staining the gels with Coomassie blue dye. The phosphorylation of the substrates was quantified by excising gel pieces corresponding to the stained substrate and counting in a scintillation counter.

[c] Represents the activity in the presence of EGTA–EDTA buffer alone.

[d] Cal.C, calphostin C.

radiolabeled substrates were excised and counted to determine the extent of phosphorylation.

As indicated in Table 1 and shown in Figure 4, maximum phosphorylation of both histone H1 and α-peptide was attained only when all three cofactors, namely, calcium, TPA, and PS, were present in the assay (Figures 4A and 4C, lanes 8). There was a five- to sevenfold increase in the phosphorylation level compared with only calcium present in the assay (Table 1, compare lane 8 with lane 2). However, the presence of calcium alone in the assay mixture resulted in a 1.5-fold increase in phosphorylation compared with the buffer alone (Table 1, compare lane 2 with lane 1). The specificity of the in vitro phosphorylation assay was demonstrated by the fact that neither 4αTPA nor phosphatidylcholine (PC) could substitute for TPA and PS, respectively, to support the full activity of the enzyme (Table 1, compare lanes 10 and 11 with lane 8). More critically, however, pretreatment of the particulate fraction with the PKC inhibitor calphostin C mitigated the maximum phosphorylation of both histone H1 and α-peptide that was obtained when all of the three cofactors were present in the assay (Table 1, compare lane 9 with lane 8).

As shown in Figures 4A and 4C, the presence of calphostin C (lanes 9) in the assay reduced the phosphorylation to a level similar to that observed when only calcium and one other coactivator (Ca^{2+} and PS [lanes 5] or Ca^{2+} and TPA [lanes 6]) were present in the assay. However, the omission of calcium had no apparent effect on the level of phosphorylation of the substrates with either PS (Figures 4A and 4C, lanes 3) or TPA (Figures 4A and 4C, lanes 4), or even the combined presence of PS and TPA (Figures 4A and 4C, lanes 7) in the assay. This suggested that other classes such as the novel and the atypical isoenzymes of PKC may not be present in the particulate extracts of fresh potato tubers. However, a minor contamination by calcium-dependent protein kinases might explain the activation obtained by calcium alone (Figures 4A and 4C, lanes 2).

Immunological Confirmation of PKC Isoforms in Potato Tubers

Analysis of cDNAs of the conventional isoforms of PKC isolated from diverse sources, including rat, mouse, rabbit, and human, revealed an overall sequence similarity in two major domains, that is, the catalytic domain and the phospholipid/DAG or the phorbol ester binding domain (reviewed in Dekker and Parker, 1994). On this basis, using affinity-purified polyclonal antibodies raised against PKC, we confirmed the presence of PKC-like enzyme in the same particulate fraction that was used to demonstrate the kinase activity in Figure 4. The antibodies are directed against a short peptide conserved in the catalytic domain of the conventional isoforms of human PKC (α, βI, βII, and γ). Results shown in Figure 5 demonstrate that the antibody recognized a protein of ~78 kD in the particulate fraction of fresh tubers (lane 1). A comigrating band was also revealed in the total cell extract of rat spleen (Figure 5, lane 2). These results are in agreement with previous studies suggesting that PKCα, βI, βII, and γ in rat spleen are between 78 and 80 kD (Kosaka et al., 1988; Wetsel et al., 1992). The migration at 50 kD of other immunoreactive species seen in the extracts of rat spleen may be due to the action of a specific protease (Sorimachi et al., 1994), representing the catalytic fragment of the PKC enzyme (Wetsel et al., 1992). The specificity of the antisera is emphasized by the observation that the reactions of the antisera with the protein species in both the extracts of rat spleen and particulate fraction of fresh tubers were successfully blocked when coincubated with the immunizing peptide in the immunoblot analysis (Figure 5, lanes 3 and 4).

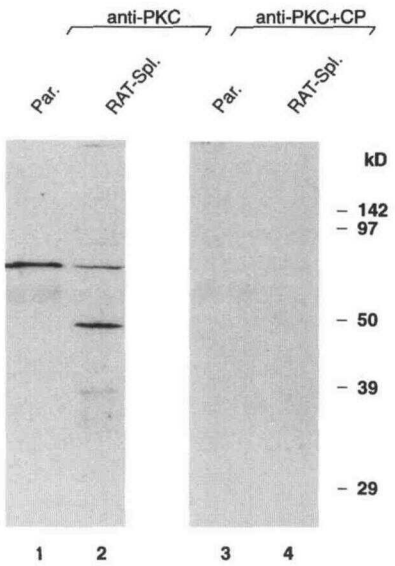

Figure 5. Confirmation of the Presence of a PKC Homolog in Potato by Immunoblotting.

Aliquots (15 μg) of total protein extracts of rat spleen (RAT-Spl.; lanes 2 and 4) and proteins from the particulate fraction of potato tubers (Par.; lanes 1 and 3) were fractionated by SDS-PAGE and immunoblotted with anti-PKC antibodies in the absence (anti-PKC) and in the presence (anti-PKC+CP) of 1.75 μM of the immunizing peptide during immunoblot analysis. The PKC antibody was raised and affinity purified against the conserved C4 catalytic domain (residues 528 to 537; PEIIAYQPYG) of the conventional isoenzymes of human PKC. Numbers at right indicate molecular mass standards in kilodaltons. Essentially identical results were obtained in five independent experiments.

The authenticity of the immunoreactive species was verified by immunoprecipitating the proteins from the particulate fraction of tubers by using the PKC-specific antibodies and subjecting them to an in-gel kinase assay. The immunoblot in Figure 6A confirmed that the protein species immunoprecipitated by the antibodies (lane 2, 78-kD band) correspond to those species observed in the particulate extracts (lane 1). The specificity of the immunoprecipitation was confirmed by the inclusion of the immunizing peptide in the immunoprecipitation reaction (Figure 6A, lane 3). The proteins from the particulate extracts and the immunoprecipitates were subjected to an in-gel kinase assay with histones as substrate. Figure 6B shows that there are at least four proteins present in the particulate extracts that phosphorylate histones (lane 4). These bands most likely correspond to different and/or breakdown products of kinases. The immunoprecipitates of the particulate extracts, on the other hand, revealed only one strongly labeled band (Figure 6B, lane 5). The specificity of this phosphorylating band was confirmed by the inclusion of the immunizing peptide in the immunoprecipitation reaction (Figure 6B, lane 6). The minor phosphorylating band observed in Figure 6B, lanes 5 and 6, may represent another kinase associated with the dominant species recognized by the PKC antibody because it was not detected in the immunoblots. However, it might also be a degradative product of the kinase(s).

The bands revealed in the in-gel kinase assay (Figure 6B) are the result of the combined effect of phosphorylation of the histone substrate and autophosphorylation of the protein kinase. To verify autophosphorylation, the dominant band observed after immunoprecipitation (Figure 6B, lane 5) was isolated from the gel and subjected to a second SDS-PAGE. Autoradiography indicated the presence of a unique radiolabeled 78-kD protein (data not shown) identical to the one seen in the immunoblot analysis. Figure 6C shows the result of the phosphoamino acid analysis of this protein, which indicated that autophosphorylation occurs on serine and threonine residues and confirmed that the immunoprecipitated kinase belongs to the serine/threonine family of kinases.

The criteria that were used to determine that the kinase characterized biochemically and shown in Figure 4 belonged to the conventional isoforms of PKC were also used with the kinase immunoprecipitated by the PKC-specific antibodies. Figure 6D shows that treatment with calphostin C led to a marked decrease in the activity of the kinase in the gel (compare lane 9 with lane 8). However, unlike the kinase characterized in Figure 4, we were unable to augment the activity of the kinase in the gel by the addition of calcium, PS, and TPA, presumably because of the inaccessibility of the lipid cofactors into the gel matrix. Therefore, we eluted the renatured kinase from the gel and observed the effects of cofactors on the kinase to phosphorylate α-peptide in vitro. As shown in Figure 6E, the addition of the cofactors PS and TPA led to a two- to threefold increase in the phosphorylation of this PKC-specific substrate (α-Pept.; compare lane 11 with lane 10). These results strongly suggest that the kinase detected by the PKC-specific antibodies corresponds to the kinase characterized biochemically in Figure 4 because both enzymes share properties common to the conventional isoforms of PKC.

It is known that phorbol esters, because of their metabolic stability, prolong the activation of PKC. This results in the eventual downregulation (depletion) of PKC, a characteristic associated with this enzyme (Huang et al., 1989). The immunoblot in Figure 7 shows that the level of the 78-kD protein kinase was noticeably increased after a 48-hr exposure of the potato tubers to TPA (lanes TPA_{500} at 48 hr) compared with the control discs (lanes W at 48 hr). However, an additional 24-hr exposure to TPA led to a significant decrease in the level of the protein (compare lanes W at 72 hr and lanes TPA_{500} at 72 hr). This suggested that prolonging TPA activation of this kinase in potato also led to its eventual downregulation, characteristically similar to what is observed with PKC in the animal cells.

Figure 7 also shows a comparable effect of prolonged exposure to TPA on PR-10a protein accumulation. This effect, however, was not observed with other PR proteins, such as chitinase and 1,3-β-glucanase. This strong correlation be-

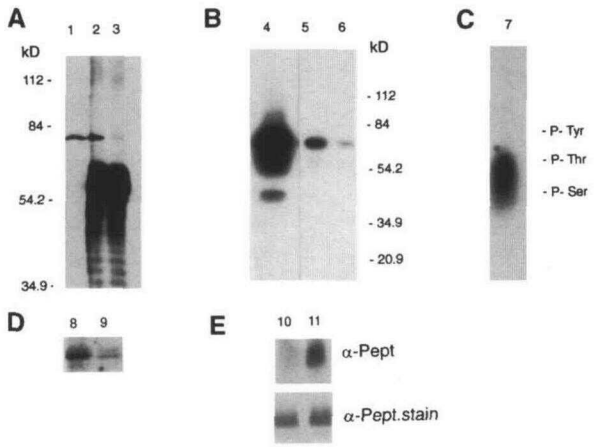

Figure 6. Analysis of the Immunoprecipitates with PKC-Specific Antibodies.

Immunoprecipitation of proteins from the particulate fraction of the potato tubers was performed as described in Methods. The nonimmunoprecipitated and the immunoprecipitated samples were fractionated by SDS-PAGE.

(A) Immunoblot analysis using the PKC-specific antibodies. An aliquot (30 μg of protein) from the particulate fraction (lane 1) and the immunoprecipitates with the PKC-specific antibodies in the absence (lane 2) and presence (lane 3) of the immunizing peptide (5 μM) in the immunoprecipitation reaction was separated by SDS-PAGE and immunoblotted with PKC-specific antibodies. Intense bands at 54 kD represent IgG. Numbers at left indicate molecular mass standards in kilodaltons.

(B) Autoradiography of the in-gel kinase assay. An aliquot (3 μg of protein) from the particulate fraction (lane 4) and the immunoprecipitates in the absence (lane 5) and presence (lane 6) of the immunizing peptide was subjected to an in-gel kinase assay as described in Methods. This is representative of at least five independent experiments. Numbers at right indicate molecular mass standards in kilodaltons.

(C) Phosphoamino acid analysis of the radiolabeled immunoprecipitate in **(B)**. The autophosphorylated protein kinase (**[B]**, lane 5) was separated from histones by SDS-PAGE. The ^{32}P-labeled immunoprecipitate was hydrolyzed with HCl and subjected to one-dimensional thin-layer chromatography as described in Methods. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) standards are as indicated.

(D) Autoradiography of an in-gel kinase assay after calphostin C treatment. After the immunoprecipitated proteins in the gel were renatured, the gels were treated (lane 9) or not treated (lane 8) with 1 μM calphostin C, as described in Methods. The in-gel kinase assay was performed as described in Methods. This is representative of three independent experiments.

(E) PS and TPA increase phosphorylation of the α-peptide. The kinase immunoprecipitated by the PKC-specific antibodies was eluted as described in Methods. PKC activity was measured with the PKC-specific substrate α-peptide (10 μM) as described in Methods. Lane 10 is a control reaction with calcium alone, and lane 11 represents the effect of calcium plus PS and TPA. The reaction mixture was separated by SDS-PAGE, and the labeled α-peptide was visualized by autoradiography (α-Pept). Relative loading was monitored by Coomassie blue staining (α-Pept.stain). This is representative of three independent experiments.

tween TPA treatment and the effect on PR-10a accumulation underscores the specificity of the signaling pathway leading to *PR-10a* activation. Overall, the behaviors of this 78-kD protein kinase immunologically and in combination with the biochemical data reinforce our proposition that a PKC-like kinase is present in potato tubers and that it participates in the pathway leading to *PR-10a* activation.

Acquired Resistance to *P. infestans* after Elicitor Treatment Is Mediated through PKC

Treatment of potato tuber discs with the elicitor arachidonic acid completely prevents fungal growth when they are infected with the compatible races of the fungus *P. infestans* (Bostock et al., 1981). It is likely that the resistance to the fungus involves a plethora of genes induced by the elicitor. We have already demonstrated that one of the induced genes, *PR-10a*, was markedly affected by modulators of PKC activity (Figures 1 and 2). We were interested to see whether the resistance to the fungus induced by the elicitor was similarly affected by the same modulators of PKC function. Therefore, we inoculated the tuber discs with zoospores of a compatible race of *P. infestans* and assessed the infection by an ELISA designed for the quantification of *Phytophthora* species.

Figure 8 shows that as expected, the application of the elicitor on the tuber discs prevented growth of the pathogen (compare bars W and AA). However, pretreatment of the discs with the PKC inhibitors calphostin C or bisindolylmaleimide before the application of arachidonic acid abolished the effect of the elicitor and restored the growth of the fungus

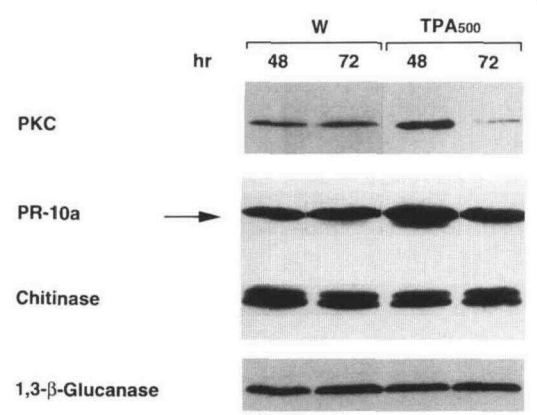

Figure 7. Immunoblot Showing the Effects of Chronic Exposure to TPA on the PKC-like Kinase, PR-10a, Chitinase, and 1,3-β-Glucanase.

After a 6-hr aging period, potato discs were treated with 1% DMSO (W) or 500 nM TPA (TPA_{500}) and incubated in the dark at room temperature. Proteins were extracted from the treated tuber discs after 48 and 72 hr, respectively. Aliquots (10 μg) from each sample were fractionated using SDS-PAGE and immunoblotted with antibodies as indicated. The arrow indicates PR-10a. Essentially identical results were obtained in three independent experiments.

to a level similar to that of the untreated discs (bar W). On the other hand, the PKC activator TPA mimicked the action of the elicitor and completely prevented infection by *P. infestans.* At the same concentrations, neither calphostin C nor TPA interfered with the normal growth of the fungus, as confirmed by the ability of the fungus to grow on rye agar plates impregnated with either TPA or calphostin C (data not shown). These results suggest that the PKC homolog present in potato tubers plays an important role in the defense response signaling pathway. The results also validate the usefulness of *PR-10a* as a marker for the defense response.

DISCUSSION

Many lines of evidence have indicated a role for protein kinases as mediators of the elicitor response (reviewed in Dixon et al., 1994; Després et al., 1995). Recently, Suzuki and Shinshi (1995) identified a kinase in tobacco cells whose activity increases in response to elicitors. Inhibitors of protein kinases have been used successfully to block this response and in particular the induction of defense genes (Raz and Fluhr, 1993; Després et al., 1995). In our study of elicitor-mediated responses in potato, we have identified a kinase with properties similar to the conventional isoforms of mammalian PKC.

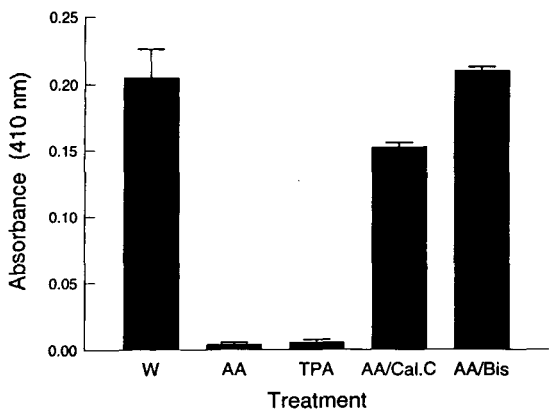

Figure 8. Acquired Resistance to *P. infestans* Is Mediated through a PKC Homolog in Potato.

The potato discs were aged for 6 hr and treated with 1% DMSO (W), arachidonic acid plus 1% DMSO (AA), 100 nM TPA, a combination of 500 nM calphostin C and arachidonic acid (AA/Cal.C), and a combination of 50 nM bisindolylmaleimide and arachidonic acid (AA/Bis). The discs were treated with the compounds for 30 min before the application of the elicitor arachidonic acid. After the elicitor was absorbed, the potato discs were inoculated with 3000 zoospores of *P. infestans* (race 1,2,3,4) and incubated for 6 days at 15°C in the dark. Quantification of the fungal mycelial growth on the inoculated discs was done by ELISA by using a *Phytophthora* detection kit (Sigma). The data are from two independent experiments, and the standard error range is indicated above each bar.

In this study, pharmacological agents with different molecular structure and modes of action against PKC were shown to affect the accumulation of PR-10a in potato. For example, calphostin C, which inactivates PKC by interacting with the regulatory domain of the enzyme (Bruns et al., 1991; Rotenberg et al., 1995), greatly reduced the elicitor-induced accumulation of PR-10a and was also able to inhibit the activity of the kinase immunoprecipitated by the PKC-specific antibodies in gel. These results, in conjunction with the biochemical characterization of the kinase, provide strong evidence for the presence of this enzyme in potato. Furthermore, the results of the EMSA and ELISA analyses suggest that PKC is not only part of the signaling cascade leading to the activation of *PR-10a* but may also play a crucial role in the overall defense response in potato.

Unlike its counterpart in the animal system, PKC in plants is not well characterized. Although some earlier reports have suggested the presence of this enzyme in plants (Oláh and Kiss, 1986; Elliott et al., 1988; Karibe et al., 1995), only recently has its activity been clearly demonstrated. Nanmori et al. (1994) partially purified an enzyme from *Brassica campestris* that exhibited all of the properties of the conventional type of mammalian PKC. When activated by the cofactors DAG or phorbol ester, calcium, and PS, the partially purified enzyme was able to phosphorylate the PKC-specific substrate α-peptide in vitro.

With the use of specific pharmacological agents, the potential biological role of this type of kinase in plants is beginning to emerge. Recently, lipid-activated kinases have been implicated in cell division (Wolniak and Larsen, 1995) and in the control of the plasma membrane proton ATPase. This pump was shown to be activated in response to a race-specific elicitor of *Cladosporium fulvum* in tomato suspension cells (Xing et al., 1996). With the use of specific inhibitors and activators of PKC, the function of the proton ATPase was shown to be controlled by the action of a PKC-like kinase. Although the link between the kinase and disease resistance was not established, the study results nevertheless suggested that perturbing the activity of this enzyme could have adverse effects on components involved in the plant defense response.

With the initial characterization of PKC as a calcium- and phospholipid-dependent protein kinase, it has since been amply demonstrated in animal cells that these kinases are activated by turnover products of cellular glycerolipids. At least for the conventional isoforms of PKC, the model of activation includes generation of inositol phosphates and DAGs through hydrolysis of plasma membrane–associated phosphoinositides by phospholipase C. DAGs are well-known activators of PKC in vivo, and it is hypothesized that for a long-term cellular response by PKC, a sustained level of this lipid is required. In animal cells, this is achieved by hydrolysis of PC by phospholipase D (Nishizuka, 1995). Interestingly,

both PC-specific phospholipase D and phosphoinositol-specific phospholipase C are present in plants, and their gene has been cloned (Hirayama et al., 1995; Shi et al., 1995; Yamamoto et al., 1995). Phospholipase C is activated in soybean cells when exposed to a polygalacturonic acid elicitor, suggesting that phosphoinositides may participate as second messengers (Legendre et al., 1993; Chandra et al., 1996). Recently, Young et al. (1996) observed that phospholipase D accumulates around the pathogen ingress site during a resistance response in rice.

Other lines of evidence suggest that in animals, free unsaturated fatty acids such as arachidonic acid can directly stimulate certain isoforms of PKC (Noar et al., 1988). Although the mechanism of this activation is not clear, the physiological role of fatty acids in the activation of PKC isoenzymes is becoming increasingly relevant (Blobe et al., 1994). Van der Hoeven et al. (1996) recently reported that *cis*-unsaturated fatty acids, including arachidonic acid, activate a protein kinase in oat root plasma membranes. This activation is mitigated by calphostin C, suggesting that a PKC-like kinase may be affected by arachidonic acid, similar to what has been observed in animal cells.

This observation is significant in light of the fact that in our system, the defense response was induced by the same lipid. It is conceivable that the application of arachidonic acid to the potato tuber discs may directly activate a PKC-like enzyme, thereby triggering a whole new set of cascades that eventually result in disease resistance. Accordingly, experiments are under way to ascertain the interaction of arachidonic acid with the PKC-like kinase and the concomitant activation of the enzyme in potato tuber extracts. It will also be interesting to explore the link between this kinase and the MAP kinase–like enzyme identified in tobacco suspension cells (Suzuki and Shinshi, 1995). Because MAP kinases can be activated through the action of PKC in mammalian and other cells (Kolch et al., 1993), it would be interesting to determine whether a similar group of MAP kinases is activated in potato and thus participates in a signaling cascade to induce resistance.

METHODS

Materials

Potato (*Solanum tuberosum* cv Kennebec) tubers were obtained from the Québec Ministry of Agriculture Les Buissons Research Station (Pointe-aux-Outardes, Québec, Canada). They were stored in the dark at 4°C and brought to room temperature 24 hr before use. Arachidonic acid, sphingosine, palmitoyl sphingosine, phosphatidylserine (PS), and phosphatidylcholine (PC) were purchased from Sigma. Arachidonic acid, PS, and PC were dissolved in chloroform and stored at −70°C. Calphostin C and bisindolylmaleimide were purchased from Calbiochem (La Jolla, CA). 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 4α-12-*O*-tetradecanoylphorbol 13-acetate (4αTPA), histone H1, and α-peptide ([ser^{25}]PKC[19-31]; RFARKGSLRQKNV) were purchased from Gibco Bethesda Research Laboratories (Burlington, Ontario, Canada). TPA, 4αTPA, and all of the inhibitors were dissolved in 100% DMSO and stored in small aliquots at −20°C. γ-^{32}P-ATP (4500 Ci/mmol) was obtained from ICN (Mississauga, Ontario, Canada). The anti–protein kinase C (PKC) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Treatments of Tuber Discs for Immunoblot Analyses

After a 6-hr aging period, tuber discs were treated with 20 μL of PKC activator and inhibitors in 1% DMSO or with a solution of 1% DMSO as control. After 30 min, the discs were treated with 20 μL of 1 μg/μL emulsion of arachidonic acid in water. The treated discs were incubated at room temperature in the dark for 48 hr. Total proteins were extracted from fresh and treated discs, separated on 14% (for pathogenesis-related protein PR-10a), 10% (for PKC), and 12% (for chitinase and glucanase) SDS–polyacrylamide gels, and blotted onto nitrocellulose membranes as previously described (Constabel and Brisson, 1992). The blots were stained with Ponceau S, blocked, and incubated with anti–PR-10a antibodies, as described previously (Constabel and Brisson, 1992). The anti-PKC antibody was used at 100 ng/mL. The antibodies raised against potato chitinase (class I) and 1,3-β-glucanase (class I) were used at a dilution of $1/5000$. The blots were then developed with the electrochemiluminescent detection system (Amersham International), according to the manufacturer's instructions. Protein quantification was performed using a protein assay (Bio-Rad) with BSA as a standard.

Preparation of Crude Nuclear Extracts and Electrophoretic Mobility Shift Assay

Treatment of potato tubers, extraction of nuclear proteins, and use of probes and gel conditions for the electrophoretic mobility shift assay (EMSA) were identical to the procedures described previously (Després et al., 1995).

Preparation of the Particulate Fraction from Potato Tubers

Potatoes were taken from cold storage, and 60 g of sliced tubers was homogenized by two 45-sec bursts in a blender in 120 mL of buffer A (50 mM Tris-HCl, pH 7.5, containing 300 mM sorbitol, 100 mM $CaCl_2$, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 30 mM β-mercaptoethanol). The homogenate was filtered through Whatman No. 1 MM paper. The filtrate was centrifuged for 10 min at 5000*g*, and the supernatant was centrifuged for an additional 1 hr at 100,000*g* at 15°C. The membrane pellet was gently resuspended in 100 mL of buffer A by 10 strokes in a Dounce homogenizer (Wheaton Scientific, Millville, NJ) and then centrifuged for 1 hr at 100,000*g* at 4°C as before. The washing procedure was repeated three times. In the last two washes, calcium was excluded from the buffer. The pellet was resuspended in 10 mL of ice-cold buffer B (50 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 50 mM NaCl, 0.5% Triton X-100, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 30 mM β-mercaptoethanol), incubated with gentle shaking at 4°C for 1 hr, and then centrifuged at 4°C, as before. The supernatant was stored at 4°C, and an

aliquot (100 μg of protein) was treated with 25 μL of Affigel protein A–agarose (Bio-Rad) for 1 hr before it was used in the kinase assay.

PKC Assay

PKC activity was quantitated by measuring incorporation of phosphorus-32 into the substrates histone H1 or α-peptide. The reaction mixture (50 μL) contained 25 μL of the particulate fraction (5 μg of protein), 20 mM $MgCl_2$, 4.95 mM $CaCl_2$, 20 μM γ-^{32}P-ATP (1 μCi per assay), 200 μg/mL histone H1 or 10 μM α-peptide, 20 μg/mL PS or PC, and 10 nM TPA or 4αTPA. Phospholipids in chloroform were evaporated to dryness under nitrogen and resuspended in 20 mM Tris-Cl, pH 7.5, with sonication and vortexing. The reaction was started with the addition of the solution of $MgCl_2$, γ-^{32}P-ATP, substrate, and $CaCl_2$ to the incubation mixture containing the particulate fraction, the phospholipid, and TPA. The final concentration of DMSO was 0.002%. After 10 min at 30°C, the reaction was stopped by the addition of 25 μL of 3 × sample buffer (150 mM Tris-HCl, pH 6.8, 6% SDS, 0.3% bromophenol blue, 30% glycerol, and 50 mM β-mercaptoethanol). An aliquot (15 μL) of the reaction mixture was separated by 12% SDS-PAGE for histone H1 and 18% for α-peptide. The phosphorylated substrates were visualized by autoradiography. For quantifiying the phosphorylated substrates, the gels were stained with Coomassie Brilliant Blue R 250, and gel slices were cut corresponding to the substrate and were subjected to scintillation counting.

The renatured kinase was excised from the gel and eluted overnight in 30 mM Hepes-HCl, pH 7.5, at 4°C. An aliquot of the eluate was used to determine PKC activity by phosphorylation of the α-peptide as described above, except that the reaction volume was 250 μL and $CaCl_2$ was adjusted to 20 μM.

Immunoprecipitation, In-Gel Kinase Assay, and Phosphoamino Acid Analysis

For immunoprecipitation, PKC-specific antibody was bound to protein G–agarose (Sigma). This was achieved by incubating 500 ng of the antibody with 30 μL of protein G–agarose overnight at 4°C. The conjugate was washed two times in buffer C (50 mM Tris-HCl, pH 7.5, 2 μg/mL aprotinin, and 2 μg/mL leupeptin). An aliquot (600 μg of protein) of the particulate extract was then added to the PKC antibody–protein G–agarose conjugate and incubated with gentle agitation for 3 hr at 4°C. The immunoprecipitate was washed three times in buffer D (buffer C containing 5 mM EGTA, 2 mM EDTA, 100 mM NaCl, and 0.5% Triton X-100). The pellet was resuspended in sample buffer and boiled for 5 min, and the sample was electrophoresed for immunoblot analysis and in-gel kinase assay. The immunoblot analysis of the immunoprecipitates was performed as described above.

The in-gel kinase assay was performed using the method of Kameshita and Fujisawa (1989), with minor modifications. An aliquot (3 μg of protein) of the particulate fraction and the immunoprecipitates were electrophoresed, except that the 10% SDS–polyacrylamide gel was polymerized with 200 μg/mL of histone H1. After renaturation of the proteins in the gel, the gel was washed in Tris buffer (50 mM Tris-HCl, pH 7.5, 5 mM β-mercaptoethanol) for 30 min at room temperature. In some experiments, the gel was treated with 1 μM calphostin C for 2 hr. After several washes with Tris buffer, both the treated and the nontreated gels were incubated in the kinase buffer (Tris buffer containing 10 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM DTT, and 1 μM ATP [50 μCi/mL γ-^{32}P-ATP]) for 3 hr at room temperature. The gel was extensively washed with a solution of 5% trichloroacetic acid and 1% pyrophosphate, dried, and subjected to autoradiography.

γ-^{32}P-labeled immunoprecipitated proteins from the in-gel kinase assay were excised and subjected to a second SDS-PAGE. After blotting, the Immobilon-P membrane (Millipore Corp., Bedford, MA) was autoradiographed, and the radiolabeled protein band corresponding to the immunoprecipitate was excised. The proteins were partially hydrolyzed in 200 μL of 6 N HCl at 110°C for 2 hr (Kamps and Sefton, 1989). The supernatant was lyophilized and resuspended in 3 μL of distilled water. The sample and 2 μL of cold phosphoamino acid standards (2 mg/mL each of phosphoserine, phosphothreonine, and phosphotyrosine) were spotted onto 100-μm thin-layer cellulose chromatography plates (Sigma). The three phosphohydroxyamino acids were separated by an ascending solvent containing isobutyric acid/0.5 M NH_4OH (5:3 [v/v]; Duclos et al., 1991). The phosphorylated amino acids were visualized by autoradiography. The positions of the three phosphoamino acid standards were visualized by spraying the chromatography plates with 0.2% ninhydrin (Sigma) in absolute ethanol followed by heat treatment.

Pathogen Infection and ELISAs

The tuber discs were prepared and treated with PKC activator and inhibitors, as described above. Elicitor treatment and inoculation of the treated tubers with *Phytophthora infestans* (race 1,2,3,4) were as described before (Constabel and Brisson, 1992). The tissue was incubated on moist filter paper in Petri dishes and incubated in the dark at 15°C. After the onset of the mycelia growth, the tubers were frozen and stored at −20°C for ELISA analysis. The ELISA analysis was performed as described by Yao et al. (1995) by using a *Phytophthora* detection kit (Sigma). The ELISA values, which are proportional to the amount of pathogen in tuber extracts, were determined using a microplate reader (model MR5000; Dynatech Laboratories, Inc., Chantilly, VA) and expressed as the absorbance at 410 nm.

ACKNOWLEDGMENTS

We thank Dr. Erich Kombrink for providing the antibodies to glucanase and chitinase. We thank Dr. Gilbert Banville for providing certified potato tubers. This work was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada and from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche, Québec, Canada.

Received November 4, 1996; accepted February 14, 1997.

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